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A THESIS

*entitled*

MECHANISTIC ASPECTS OF THE MONOOXYGENASE SYSTEM

FROM *Methylococcus carosulatus*

*by*

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University of Warwick

In partial fulfilment of the  
requirements for the degree of  
Doctor of Philosophy at the  
University of Warwick

Department of Chemistry and Molecular Sciences

June 1982

*This thesis is dedicated to  
Mom and Bill, also my wife  
Bronwen, without whose  
encouragement and support  
this work would not have  
been undertaken.*

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\*\*\*

DECLARATION

The work presented herein has been carried out in the laboratories of the Department of Chemistry and Molecular Sciences at the University of Warwick under the supervision of Dr. B. T. Golding and is thought to be original except where due and proper acknowledgement has been made.

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### SUMMARY

The soluble nature of the monooxygenase system from *Methylococcus capsulatus* (Bath) makes it an ideal monooxygenase enzyme for mechanistic studies.

Oxidation of substituted aromatic substrates gave products that were oxidised in the para-position of the ring, the efficiency of the process being dependent upon the size of the substituent group. During these oxidations of aromatic substrates products resulting from an 'NIH' shift were observed. In the case of naphthalene, this led to substantial quantities of 2-naphthol in addition to the more usual product, 1-naphthol, observed with this type of enzyme.

Substituted aromatic compounds were also oxidised on the side chain, predominantly  $\alpha$  to the aromatic ring, with  $\beta$ -methylstyrene providing the only exception. The efficiency of the oxidation of the aromatic side-chain was also found to be dependent on the nature of the substituent group.

Oxidation of cyclopropane to cyclopropanol, of methylcyclopropane to cyclopropylcarbinol and of olefinic groups with retention of configuration suggests an oxenoid mechanism.

\*\*\*\*\*

# ABBREVIATIONS

1.	9BBN	9-Bora-bicyclo[3.3.1]nonane
2.	h	hour
3.	min.	minute
4.	t-BuOK	potassium tertiary butoxide
5.	t-BuOH	tertiary butanol
6.	DMSO	dimethylsulphoxide
7.	D.N.P.	dinitrophenylhydrazine
8.	TLC	thin layer chromatography
9.	U.V.	ultra violet
10.	m.p.	melting point
11.	p.p.m.	parts per million
12.	h.p.l.c.	high pressure liquid chromatography
13.	GLC	gas liquid chromatography
14.	m/z	mass to charge ratio
15.	NMR	nuclear magnetic resonance
16.	THF	tetrahydrofuran
17.	R <sub>f</sub>	retardation factor
18.	b.p.	boiling point
19.	NADH	nicotinamide adenine dinucleotide
20.	A.R.	analytical reagent

\*\*\*\*\*

#### NON-SYSTEMATIC NOMENCLATURE

1.	Aroclor	a commercially available poly-chlorinated biphenyl
2.	Acetanilide	N-phenylacetamide
3.	Toluene	methylbenzene
4.	Cresol	methylphenol
5.	Styrene	ethenylbenzene
6.	Styrene epoxide	phenyloxirane
7.	Cumene	(1-methylethyl)-benzene
8.	Cinnamyl alcohol	3-phenyl-2-propen-1-ol
9.	Ethylanisole	(4-ethyl)methoxybenzene
10.	Cinnamaldehyde	3-phenyl-2-propenal
11.	$\alpha$ -Methylstyrene	(1-methylethenyl)benzene
12.	Propylene oxide	methyloxirane
13.	N-propargylvaleramide	N-propynyl pentanamide
14.	Propargyl	propynyl
15.	Valeric anhydride	pentanoic anhydride
16.	Diketene	2-oxetanone, 4-methylene
17.	$\beta$ -Methylstyrene	(2-methylethenyl)benzene

\*\*\*\*\*

PUBLICATION

H. Dalton, B. T. Golding, B. W. Waters

R. Higgins and J. T. Taylor,

*J. Chem. Soc. Chem. Comm.*, 1981, 482

Oxidations of cyclopropane, methylcyclo-  
propane and arenes with the monooxygenase  
system from *Methylococcus capsulatus*

\*\*\*\*



## CHAPTER 1

### INTRODUCTION

#### 1.1 MONOOXYGENASE ENZYMES

The reaction of oxygen with organic molecules is normally exothermic and given that ca. 20% of the earth's atmosphere is oxygen it is thermodynamically surprising that strict aerobes such as *homo sapiens* have evolved. What has made this possible is the fact that under normal conditions the reaction of molecular oxygen with organic compounds is under kinetic, not thermodynamic control. Molecular oxygen has a triplet ground state, i.e. it exists as a diradical, whereas the majority of organic compounds exist as singlets. The reaction of a triplet molecule with a singlet to give singlet products is a spin forbidden process and as such does not readily occur<sup>1</sup>.

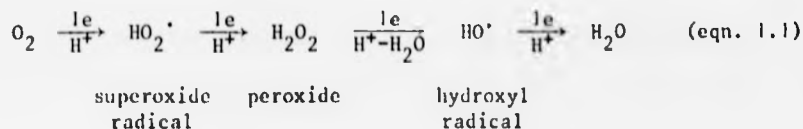
Oxygenases are enzymes that have, by various mechanisms, overcome this kinetic energy barrier and catalyse the insertion of oxygen into organic substrates. Broadly speaking, there are two types of oxygenase enzymes:

1. The dioxygenases are enzymes that effect the incorporation of both atoms of molecular oxygen into the same substrate, e.g., pyruvate carboxylase (E.C. 1.13.11.1).
2. Monooxygenases, which the rest of this thesis will be concerned with, are sometimes called mixed-function oxygenases because they catalyse the incorporation of one atom of molecular oxygen

into an organic substrate whilst the second atom is reduced to water.

Initially it was believed that the oxygen atom that was incorporated into the substrate was derived from a water molecule. Evidence that it was derived from molecular oxygen and not water was gained in 1955 by two groups working independently. Hayaishi<sup>2</sup> used pyrocatechase (E.C. 1.13.11.1), while Mason<sup>3</sup> used a phenolase complex. Both incubated the substrate with the enzyme in a buffer containing  $\text{H}_2^{16}\text{O}$  in the presence of  $^{18}\text{O}_2$ . A similar experiment using  $\text{H}_2^{18}\text{O}$  in the presence of  $^{16}\text{O}_2$  provided a control experiment. In all cases it was found that  $^{18}\text{O}$  was only incorporated when present as  $^{18}\text{O}_2$  and not when present as  $\text{H}_2^{18}\text{O}$ , thus demonstrating that the incorporated oxygen is supplied by molecular oxygen.

As has already been pointed out the direct reaction of molecular oxygen with an organic substrate is a spin forbidden process. To effect such oxidations a monooxygenase enzyme must be able to 'activate', in some way, the oxygen molecule. The nature of this 'activated oxygen complex' has been the subject of intense research ever since the realisation that molecular oxygen was involved. There are two ways in which this activation could be theoretically achieved: one is by oxidation of the oxygen molecule to ozone, but biological systems do not produce ozone<sup>4</sup> and so this method has been discounted; the alternative is reduction, for which four reducing equivalents are required to effect complete reduction of a molecule of oxygen *via* the reactive intermediates shown in eqn. 1.1

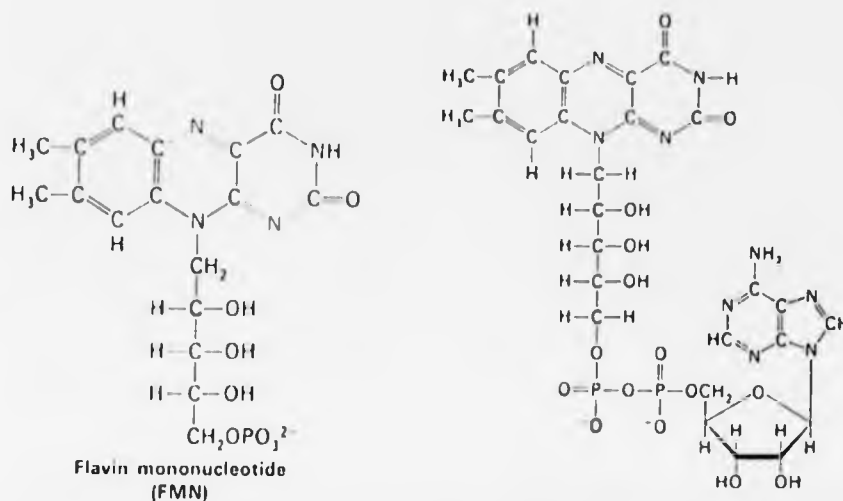


Whether these intermediates exist as shown or as their anions will depend on the pH of the medium. Of the intermediates shown, only the hydroxyl radical and peroxide have a sufficiently high redox potential to oxidise unactivated hydrocarbons. If free peroxide, produced by the enzyme, were the hydroxylating agent, then inclusion of catalase should prevent oxygenation. Similarly, the use of superoxide dismutase would show if superoxide were used. Note that these enzymes have no effect on oxidations mediated by *Methylococcus capsulatus* (Bath)<sup>5</sup>. It is unlikely that the hydroxyl radical is used, because it is such a reactive entity that it would be unable to produce the specificities shown by these enzymes. Also, it would probably react with the enzyme itself.

To overcome the kinetic barrier to reaction by molecular oxygen, monooxygenase enzymes have developed a number of different methods that will be outlined here, and in some cases, presented more fully in subsequent sections. The methods used by the different monooxygenases have led to a further sub-division of these enzymes based on the cofactor that is used.

#### 1.1.1 Flavoprotein Monooxygenases<sup>6,7,8</sup>

These are enzymes that contain flavin mononucleotide (FMN Fig. 1.1) or flavin adenine dinucleotide (FAD Fig. 1.1) as their cofactor and functional group, no transition metals being present. The flavin is tightly bound to the enzyme and does not normally dissociate at the end of the catalytic cycle, hence the need for an external reductant which is normally NADH or NADPH<sup>7</sup>.



Structure of the oxidized form of flavin adenine dinucleotide (FAD).

Fig. 1.1

The oxidised flavoproteins are planar and on reduction become 'bent' with the two external rings folding up, out of the plane of the central B ring, to give what is termed the butterfly appearance. This, according to Walsh<sup>6</sup>, is advantageous because altering the shape of the flavin can change its binding constant and hence its redox potential, allowing the flavoenzyme to function with a variable redox potential.

Owing to the thermodynamic stability of the one electron reduced flavin semiquinone (Fig. 1.2), flavin cofactors are, unlike pyridine cofactors (e.g.  $\text{NAD}^+$ ), able to participate in both  $2e^-$  and  $1e^-$  redox reactions. It is this capability that enables the reduced (dihydro) form of the flavin to be autoxidised by oxygen, again unlike the pyridine cofactors, that are stable to oxygen. This forms

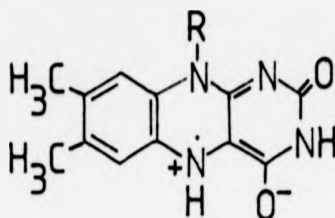


Fig. 1.2

the basis for a postulated mechanism for this group of enzymes.

A very important point in this mechanism is that all of the substrates are 'activated', i.e. they all contain a heteroatom that is used in the reaction mechanism, with hydroxylation normally taking place adjacent to this heteroatom.

The mechanism that is believed to be utilised by these enzymes is shown in Fig. 1.3. This is not universally accepted (see section on models in this Chapter). The proposed mechanism involves initial oxidation of the reduced flavin cofactor by dioxygen to give the flavin semiquinone and superoxide anion<sup>7</sup>. Recombination of these two radicals to give the 4a-hydroperoxide derivative can then take place without violating any of the principles mentioned previously for the reaction of triplet oxygen with singlet compounds to give singlet products. The 4a-hydroperoxide complex is then believed to hydroxylate the substrate, p-hydroxybenzoate in Fig. 1.3, by the mechanism shown.

Evidence for this pathway comes from a number of different sources. Kemel and Bruice<sup>7</sup> have made the crystalline N-5-ethyl-4a-hydroperoxide adduct and shown that its UV/vis spectrum is identical to that obtained in pre-steady state kinetic studies on p-hydroxybenzoate hydroxylase. In this study three spectroscopically distinct

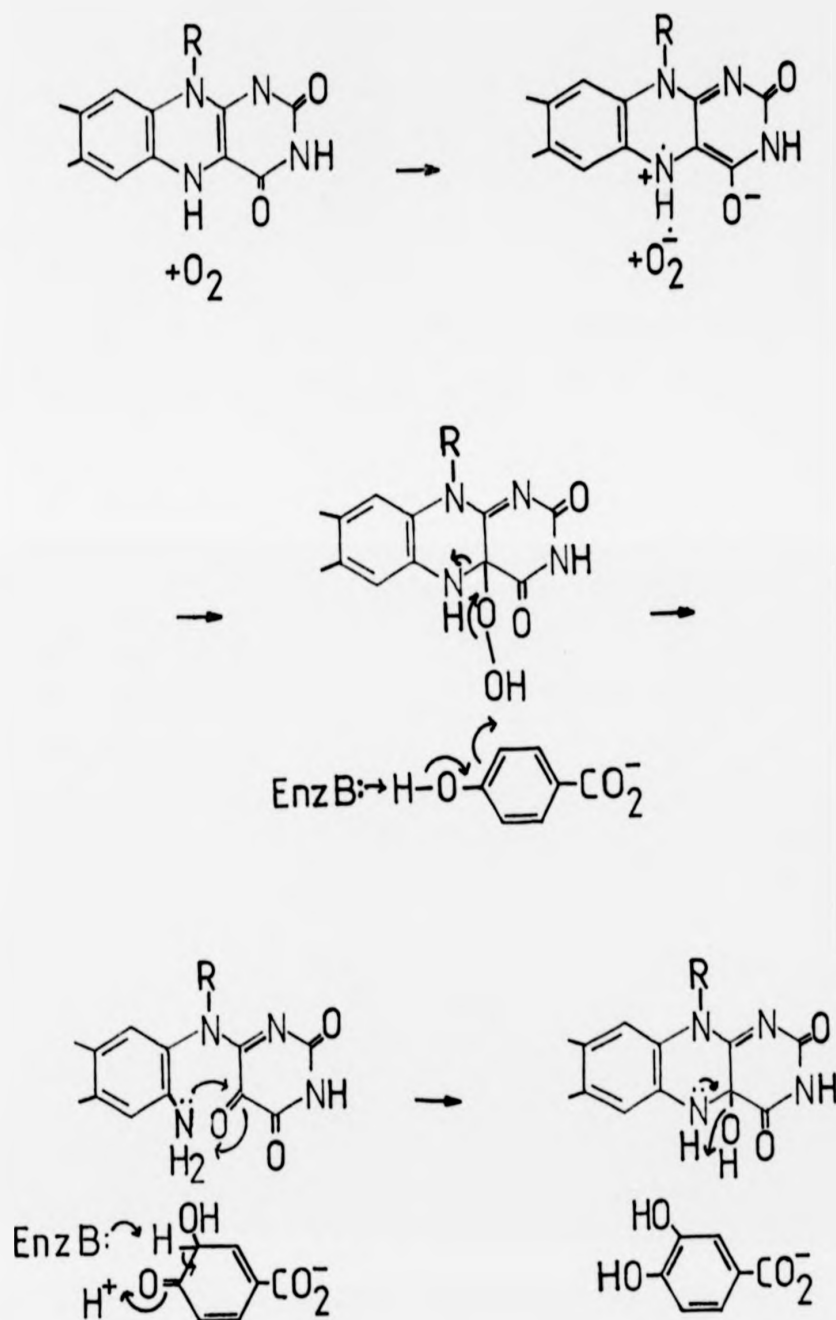


Fig. 1.3

Proposed mechanism of a flavin dependent monooxygenase enzyme.

intermediates were seen: the 4a-hydroperoxide, the corresponding 4a-hydroxy compound and a ring-opened flavin derived from this hydroxy compound. Further support for this mechanism comes from an X-ray crystallographic study that shows that the substrate in p-hydroxybenzoate hydroxylase lies with its aromatic ring along the C4-N5 edge of the flavin. This makes Hemmerich's<sup>9</sup> postulate that the oxidising species is the C10a hydroperoxide, formed *via* a dioxetane bridge with C4a hydroperoxide, seem unlikely.

#### 1.1.2 Heme-containing Monooxygenases

There are two subgroups of the heme-containing monooxygenases depending on whether the immediate reductant is a flavin or an iron-sulphur complex. Both will be dealt with in the section on cytochrome P<sub>450</sub>.

The preceding two sections represent the best understood examples of monooxygenase activities. The following two groups are not so well characterised mechanistically.

#### 1.1.3 Pterin-requiring Monooxygenases<sup>6,7</sup>

This group of enzymes contains mainly the mammalian monooxygenases that are responsible for the hydroxylation of the three aromatic aminoacids phenylalanine, tyrosine and tryptophan.

Fig. 1.4 shows that the biologically active pterin, tetrahydrobiopterine, bears some similarity to the isoalloxazine ring system of the flavins and so might be expected to function by a similar mechanism. However, analysis of the three amino acids that are substrates for these enzymes shows that they differ in one important aspect from the

flavoenzyme substrates, in that only one of these amino acids is 'activated', i.e. bears a heteroatom in a ring.

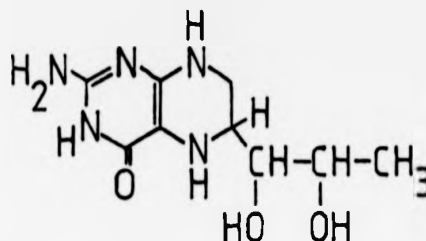


Fig. 1.4

Spectral changes are seen during the conversion of phenylalanine to tyrosine by phenylalanine hydroxylase that can be interpreted in terms of a similar mechanism to that proposed for the flavin enzymes. However, phenylalanine hydroxylase does contain two atoms of iron (the other known pterin-dependent enzymes also contain iron<sup>6</sup>) that undergo redox changes during the catalytic cycle<sup>7</sup>. Coupled with the lower reactivity of pterins towards oxygen ( $t_{1/2}$  in min.) when compared with flavins ( $t_{1/2}$  in sec.), it would appear more likely that the iron present has a function of increasing reactivity, though at present no experimental evidence is available to support this postulate.

#### 1.1.4 Copper-containing Monooxygenases<sup>7</sup>

The two best known examples of this class of enzyme are tyrosinase and dopamine- $\beta$ -hydroxylase. Available evidence suggests that these two enzymes do not share the same mechanism<sup>7</sup>. Functionally active tyrosinase is believed to be dimeric with two cuprous ions per active site that do not appear to undergo any valence changes



during the catalytic cycle. Substrate radical signals are not seen during enzymic oxidation and the requirement for a diphenol has led Hamilton to suggest the reaction scheme outlined in Fig. 1.5.

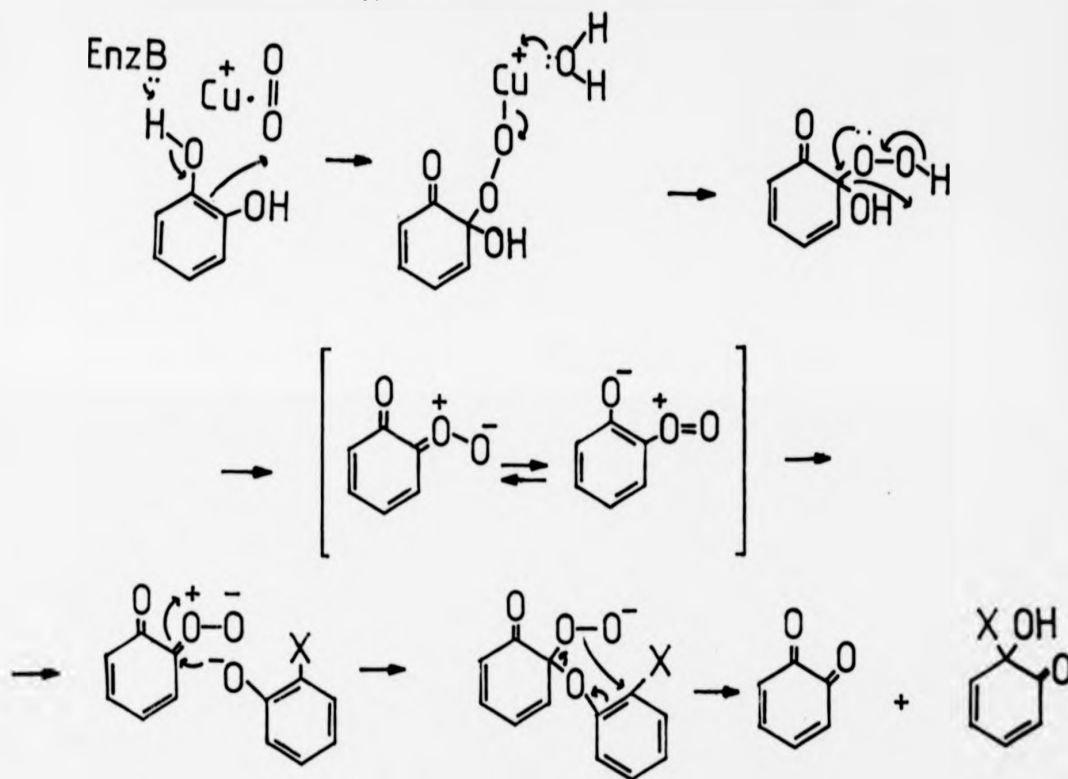


Fig. 1.5 Mechanism of tyrosinase suggested by Hamilton

Although the number of copper atoms present in a molecule of dopamine- $\beta$ -hydroxylase is not known for certain, it has long been believed that two are present and that each one is reduced from  $\text{Cu}^{2+}$  to  $\text{Cu}^+$  by ascorbate. Hamilton suggested that a  $\text{Cu}^{2+}\text{O}_2^-$  complex may then attack an acid or amine group on the enzyme to give a peracid or peramide (*cf.* Fig. 1.6) which would be the active oxygen-transfer species. Liones and Skotland<sup>10,11</sup>, however, have presented some evidence that suggests the involvement of only one copper atom; they believe that the two reducing equivalents

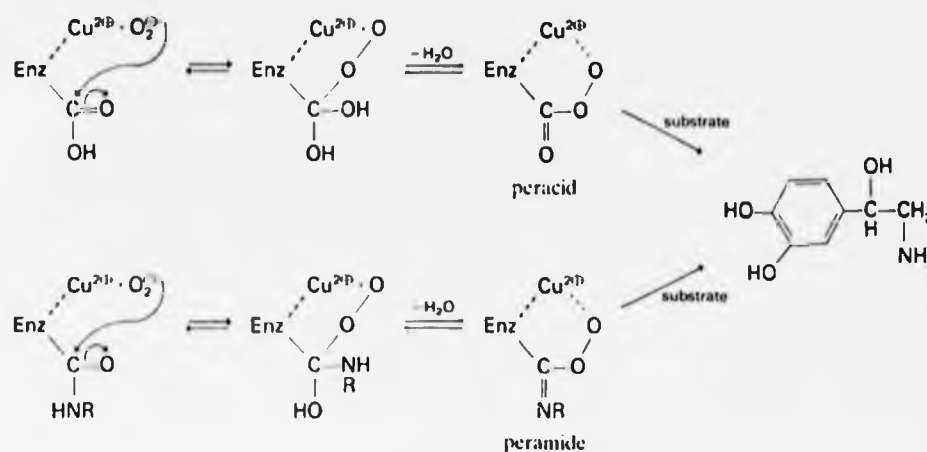
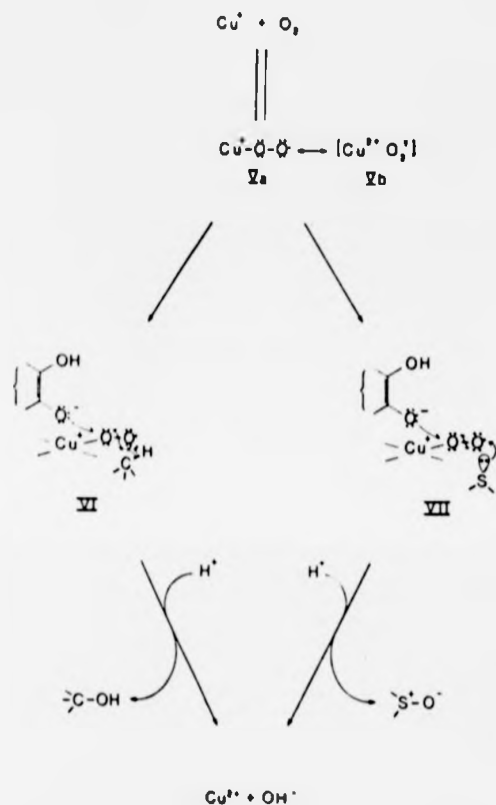


Fig. 1.6 Suggested mechanism of dopamine-β-hydroxylase

are fed into a copper-oxygen complex independently, from the ascorbate molecule. May and his co-workers<sup>12</sup> support this hypothesis and believe that the mechanism shown in Fig. 1.7 could explain their results, with hydroxylations going *via* a radical mechanism and sulphoxidations by electrophilic attack of the copper-oxygen complex on the lone pair of electrons on the sulphur.

## 1.2 BACTERIAL MONOOXYGENASE SYSTEMS

Although the title of this section suggests a general review of monooxygenase systems derived from bacterial sources, attention will be focussed mainly on the system obtained from *Methylococcus capsulatus* strain Bath (M.C.) with comparisons, where



Mechanism of dopamine- $\beta$ -hydroxylase suggested by May

Fig. 1.7

relevant, to monooxygenases derived from other organisms.

### 1.2.1 Isolation and Characterisation

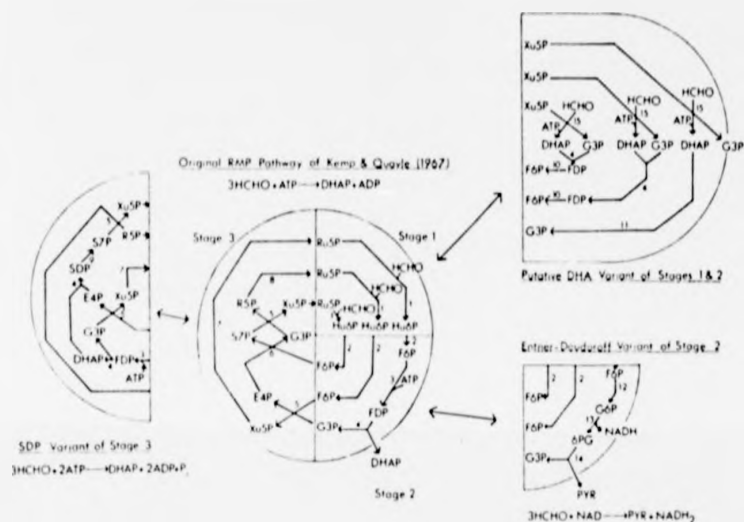
As the name suggests, the Bath strain of *Methylococcus capsulatus* was obtained from the warm water springs of the city of Bath. It was originally isolated in 1970 by Whittenbury and his co-workers<sup>13</sup>, who described it as a strictly aerobic, gram negative, non-motile cocci. It is a methylotroph in that it derives both its energy and carbon from the oxidation of methane,

though some evidence exists, to be dealt with later in this section, that it may be able to fix carbon by an autotrophic mechanism<sup>14</sup>.

A classification scheme for methane-oxidising bacteria has been put forward by Whittenbury and his co-workers<sup>14</sup> and is reproduced in Table 1.1. This shows that *Methyloccoccus capsulatus* is a type 1 organism belonging to the B subgroup and the table also indicates the morphological and biochemical determinants that place it in this group, some of which will be expanded later in this section.

Table 1.1 shows that the ribulose monophosphate pathway (shown in Scheme 1.1) is the principle method of assimilating carbon for anabolic processes in the cell. Other methods that are used are the serine pathway (*cf.* Scheme 1.2) and the autotrophic Calvin cycle (Scheme 1.3). It had previously been thought that a given organism used only one of these pathways, but the finding of ribulose 1,5-diphosphate carboxylase and phosphoribulokinase, both key enzymes in the Calvin cycle, in *Methyloccoccus capsulatus* indicates that this may not be so. It is presumptuous to assume that the presence of one or two enzymes is evidence for a functioning metabolic pathway, but the demonstration of CO<sub>2</sub>-fixation during growth on methane is seen as supportive evidence for the existence of an autotrophic pathway in M.C.<sup>14</sup>. Indeed, the authors state that the serine pathway may also be functional in this organism. The physiological relative importance of the serine and autotrophic pathways is unknown.

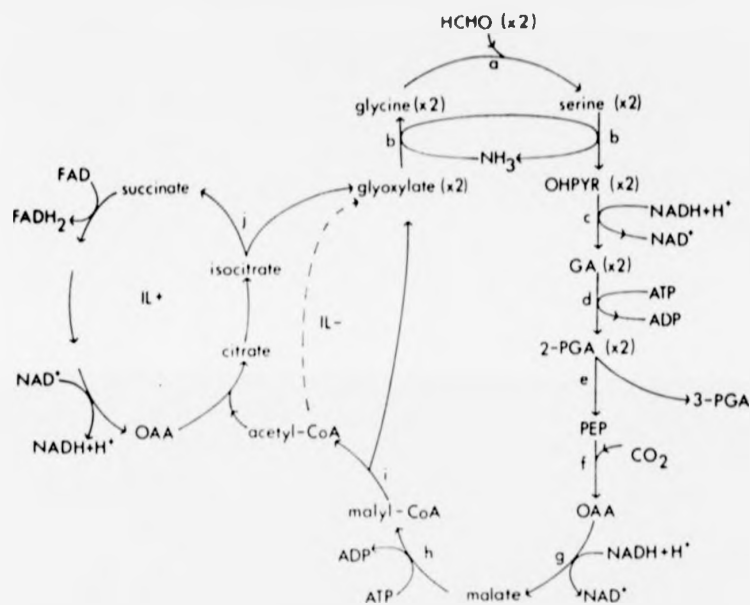
The C<sub>1</sub> units that are used in these processes arise from oxidation of methane *via* methanol to formaldehyde. The initial step in monooxygenase-mediated oxygenation has proved to be very difficult to achieve in synthetic chemistry and so a lot of research has been conducted in an attempt to determine the enzymic mechanism, in the hope that it could then be reproduced in the



RMP and its variations. Ru5P, Ribulose-5-phosphate; Hu6P, D-erythro-L-glycero-3-hexulose-6-phosphate; F6P, fructose-6-phosphate, FDP, fructose-1,6-diphosphate; G3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; Xu5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; SDP, sedoheptulose-1,7-diphosphate; R5P, ribulose-5-phosphate; G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; pyr, pyruvate; 1, 3-hexulosephosphate synthase; 2, phospho-3-hexuloseisomerase; 3, 6-phosphofructokinase (EC 2.7.1.11); 4, fructose diphosphate aldolase (EC 4.1.2.13); 5, transketotase (EC 2.2.1.1); 6, transaldolase (EC 2.2.1.2); 7, ribulose phosphate epimerase (EC 5.1.3.1); 8, ribulose phosphate isomerase (EC 5.3.1.6); 9, sedoheptulose diphosphatase; 10, fructose diphosphatase (EC 3.1.3.11); 11, triosephosphate isomerase (EC 5.3.1.1); 12, glucose phosphate isomerase (EC 5.3.1.9); 13, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); 14, 6-phosphogluconate dehydratase (EC 4.2.1.12) + phospho-2-keto-3-deoxygluconate aldolase (EC 4.1.2.14); 15, transketolase + triokinase (EC 2.7.1.30).

Scheme 1.1

Ribulose monophosphate pathway. Reproduced from J. Colby, H. Dalton, and R. Whittenbury, *Ann. Rev. Microbiol.*, 1979, **33**, 491

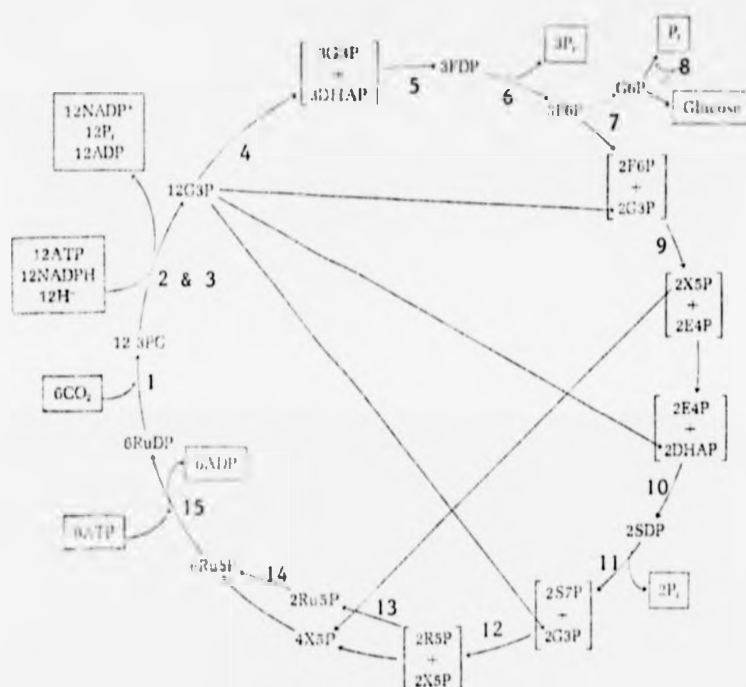


Serine pathway. *a*, Serine transhydroxymethylase (EC 2.1.2.1); *b*, serine glyoxylate amino-transferase; *c*, hydroxypyruvate reductase (EC 1.1.1.29); *d*, glyceralate kinase (EC 2.7.1.31); *e*, phosphopyruvate hydratase (EC 4.2.1.11); *f*, phosphoenol-pyruvate carboxylase (EC 4.1.1.31); *g*, malate dehydrogenase (EC 1.1.1.37); *h*, malate thiokinase (EC 6.2.1.-); *i*, malyl-CoA lyase (EC 4.1.3.24); *j*, isocitrate lyase (EC 4.1.3.1); ----, unknown reactions; OH-PYR, hydroxypyruvate; GA, glyceralate; PGA, phosphoglycerate; PEP, phosphoenolpyruvate; OAA, oxaloacetate. Net reaction (IL+):  $2\text{HCHO} + \text{CO}_2 + \text{FAD} + 2\text{NADH}_2 + 3\text{ATP} \rightarrow 3\text{-PGA} + 2\text{ADP} + 2\text{NAD} + \text{FADH}_2$ .

Scheme 1.2

Serine pathway. Reproduced from J. Colby, H. Dalton, and R. Whittenbury *Ann. Rev. Microbiol.*, 1979, 33, 495

**Scheme 1.3**      **The Calvin cycle.**



**Key:**

### Compounds

- |      |   |                               |
|------|---|-------------------------------|
| 3PG  | = | 3-phosphoglyceric acid        |
| G3P  | = | glyceraldehyde 3-phosphate    |
| DHAP | = | dihydroxyacetone phosphate    |
| FDP  | = | fructose 1,6-diphosphate      |
| F6P  | = | fructose 6-phosphate          |
| G6P  | = | glucose 6-phosphate           |
| E4P  | = | erythrose 4-phosphate         |
| X5P  | = | xylulose 5-phosphate          |
| SDP  | = | sedoheptulose 1,7-diphosphate |
| S7P  | = | sedoheptulose 7-phosphate     |
| R5P  | = | ribose 5-phosphate            |
| Ru5P | = | ribulose 5-phosphate          |
| RuDP | = | ribulose 1,5-diphosphate      |

## Scheme 1.3 (continued)

Enzymes

1. Ribulosediphosphate carboxylase (EC.4.1.1.39)
2. Phosphoglycerate kinase (EC.2.7.2.3)
3. Glyceraldehyde-phosphate dehydrogenase (NADP)  
(EC.1.2.1.9)
4. Triosephosphate isomerase (EC.5.3.1.1)
5. Fructosediphosphate aldolase (E.C.4.1.2.13)
6. Hexose diphosphatase (EC.3.1.3.11)
7. Glucosephosphate isomerase (EC.5.3.1.9)
8. Glucose-6-phosphatase (EC.3.1.3.9)
9. Transketolase (EC.2.2.1.1)
10. Fructosediphosphate aldolase (EC.4.1.2.13)
11. Hexosediphosphatase (EC.3.1.3.11)
12. Transketolase (EC.2.2.1.1)
13. Ribosephosphate isomerase (EC.5.3.1.6)
14. Ribulosephosphate-3-epimerase (EC. 5.1.3.4)
15. Phosphoribulokinase (EC.2.7.1.19)

Reproduced from Lehninger, 'Biochemistry', 2nd Edition,  
Worth Publishers Inc., 1975.



TABLE 1.1  
Tentative Classification Scheme for Methane-oxidising Bacteria<sup>a</sup>

Determinants	Type I	Type II
Membrane arrangement	Bundles of vesicular disks	Paired membranes around cell periphery
Resting stages	Cysts ( <i>Azotobacter</i> -like)	Exospores or lipid cysts
Major carbon assimilation pathway	RNP (3-hexulosephosphate synthase)	Serine pathway (hydroxypyruvate reductase + 3-hexulosephosphate synthase)
TCA cycle	Incomplete (2-oxoglutarate dehydrogenase negative)	Complete
Nitrogenase	Some +	+ (those tested)
Predominant fatty acid		18
C chain length	16	
	Subgroup A	Subgroup B
Autotrophic CO <sub>2</sub> fixation	-	+
DNA base ratio (G+C)	50-54	62.5
Isocitrate dehydrogenase	NAD or NADP dependent	NAD dependent
Cell shape	Rod & ? coccus	Coccus
Growth at 45°C	some +	+
Examples	<i>Methylobacter</i> <i>methanica</i> and <i>Methylobacter albus</i>	<i>Methylobacter</i> <i>trichosporium</i> (both obligate) and <i>Methylobacterium organophilum</i> (facultative)
	Subgroup obligate <sup>b</sup>	Subgroup facultative <sup>c</sup>

<sup>a</sup>Not all strains classifiable into type I and type II have been shown to possess all the biochemical characteristics outlined in this scheme.

<sup>b</sup>Use methanol and formaldehyde as carbon and energy source, but not C<sub>2</sub> compounds.

<sup>c</sup>Use variety of organic compounds, e.g. glucose as carbon and energy source.

Reproduced from J. Colby, H. Dalton and R. Whittenbury, *Ann. Rev. Microbiol.*, 1979, **33**, 483

laboratory. One of the major problems in attempting this line of research is the fact that most of these enzymes, e.g. M.C. strain Texas<sup>15</sup> and *Methylobacillus methanica*<sup>16</sup> are not soluble and are embedded in the cell membranes. Attempted removal of the enzyme often leads to its inactivation. The great advantage of working with the M.C. monooxygenase lies in the fact that it is a soluble enzyme. After disruption of the cell's membrane the monooxygenase can be removed by simple centrifugation followed by decantation of the supernatant liquid which contains the enzyme.

#### 1.2.2 Substrate Specificity and Electron Donors

As has already been pointed out the physiological function of the monooxygenase system derived from M.C. is believed to be oxygenation of methane to give methanol. What is surprising is the very wide range of compounds that this enzyme system will accept as substrates in addition to methane. Among other C<sub>1</sub> compounds that the enzyme system will oxidise are some halogen-substituted methanes: chloromethane was oxidised readily, with bromomethane oxidation being slower but iodomethane was not oxidised at all<sup>5</sup>. This same pattern was repeated with multiply halogen-substituted methanes, dichloromethane being oxidised more rapidly than trichloromethane, whereas tetrachloromethane was not oxidised at all. The cyano, nitro and thio derivatives of methane were oxidised and oxidation of CO gave CO<sub>2</sub><sup>5</sup> (cf. *Methylobacillus methanica*<sup>17</sup> and *Methylobacillus trichosporium*<sup>18</sup>). This decrease in oxidation rate with increasing size of the substrate is a general feature that has recurred with all of the series of substrates used (see Chapters 3 and 5). Although C<sub>1</sub>-C<sub>8</sub> n-alkanes were oxidised at the C<sub>1</sub> and C<sub>2</sub> positions there was a rapid decrease in the rate

of oxidation of substrates larger than pentane<sup>5</sup>. This is similar to the results of oxidation with *Methylobacillus methanica*<sup>19</sup>: hexane and heptane were not oxidised and the specific activity of the oxygenase decreased markedly on going from propane to butane. Although *Methylobacillus trichosporium*<sup>19</sup> does oxidise these C<sub>6</sub> and C<sub>7</sub> n-alkanes the specific activity also decreases fairly steadily over the complete range of C<sub>1</sub>-C<sub>7</sub>. As with M.C. both of these organisms give products arising from oxidation at  $\omega$  and  $\omega-1$  positions.

The oxidation of terminal alkenes has been reported to give the epoxides<sup>5</sup> only. Internal alkenes gave a mixture of the epoxides and products arising from attack at C<sub>1</sub>, both with retention of configuration. Interestingly *cis*-but-2-ene gave, in addition to the *cis*-epoxide, 2-butanone. Whether this was accompanied by an 'NIH' type of shift was not reported. It was noted that the *trans*-isomers were oxidised more rapidly than their *cis*-counterparts by M.C., but not with *Methylobacillus trichosporium* or *Methylobacillus methanica*, although in most other aspects oxidations of alkenes by these two organisms gave similar results to those obtained with M.C. For *Pseudomonas oleovorans* May and his co-workers<sup>20</sup> have found that the oxidation of *trans,trans*-1,8-dideutero-1,7-octadiene gave a product that consisted of 30% epoxide of retained configuration and 70% with inverted configuration. These were suggested to arise through rotation of an intermediate radical or zwitterion, produced by a two-step mechanism of oxidation shown in Fig. 1.8.

*Methylobacillus trichosporium*, *Methylobacillus methanica* and M.C. all oxidise dimethyl and diethyl ether. Alicyclic, heterocyclic and aromatic (see Chapter 3) compounds were only oxidised by *Methylobacillus trichosporium* and M.C.<sup>19</sup>

Tonge *et al.*<sup>18</sup> reported that the immediate electron donor

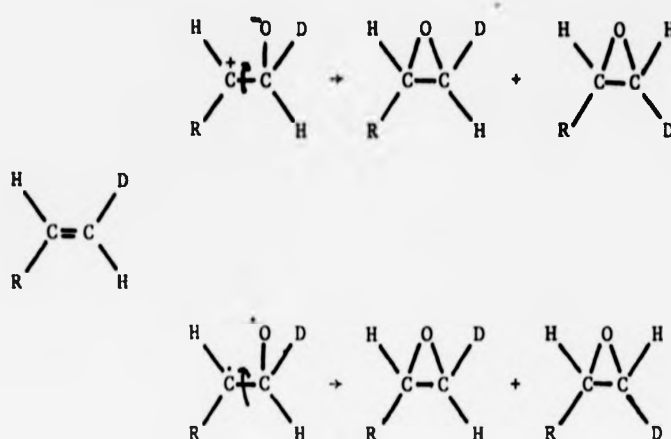


fig. 1.8

of the *Methylosinus trichosporium* monooxygenase was a cytochrome  $\text{C}_{\text{CO}}$  that could be reduced by ascorbate or by electrons derived from the reaction of methanol dehydrogenase and methanol. In crude extracts neither NADPH nor NADH could serve as reductant and it was argued that *in vivo* they may supply electrons *via* an electron-transport chain. This result could not be repeated by other workers<sup>21</sup> who found NADH to be the most effective electron donor, NADPH giving 60-100% of the activity of NADH depending on the substrate. The methanol/methanol dehydrogenase system gave only 12% of the activity of NADH and ascorbate gave no activity. This ability of an alcohol to support monooxygenase activity has also been reported for *Methylomonas methanica*<sup>17</sup>, although in this case ethanol was the reductant. For all three enzymes the most effective reductant was NADH. With *Methylomonas methanica*<sup>17</sup> NADPH was only 60% as effective as NADH and with M.C.<sup>22</sup> this value decreased to 50%.

The above discussion does show a very real similarity between the monooxygenases from *Methylosinus trichosporium*, *Methylomonas methanica* and M.C. Differences do exist in that M.C. gives a soluble

enzyme, whereas that from *Methylomonas methanica* is particulate. The enzyme from *Methylosinus trichosporium* has been reported as particulate<sup>18</sup> and soluble<sup>21</sup>. Higgins<sup>23</sup> has stated that the mono-oxygenase of *Methylosinus trichosporium* can be particulate or soluble depending on the growth conditions and this can alter the substrate specificity. He did not, however, state what the differences in growth conditions were.

The broad substrate specificity of the enzymes discussed could be interpreted as being due to the generation of a very non-specific hydroxylating agent such as superoxide anion or peroxide. This possibility is decreased by the finding that the monooxygenase enzyme from M.C. is not inhibited by superoxide dismutase or by catalase and that, unlike the cytochrome P<sub>450</sub> system, oxidations cannot be supported by sodium chlorite or sodium periodate<sup>5</sup>.

### 1.2.3 Monooxygenase Inhibitors

The susceptibility to inhibitors is one way in which the mono-oxygenase enzymes from M.C. and *Methylomonas methanica* differ. The enzyme from *Methylomonas methanica*<sup>16</sup> has been shown to be inhibited by a wide range of compounds including metal chelators, some metal ions and acetylene, whereas the M.C. enzyme has only been shown to be inhibited by 8-hydroxyquinoline and acetylene. With the enzyme of *Methylosinus trichosporium* the picture is unclear due to conflicting reports. Tonge *et al.*<sup>18</sup> showed that this enzyme was inhibited by a wide range of compounds including cyanide. Stirling<sup>21</sup>, however, found that only 8-hydroxyquinoline and acetylene inhibited methane or ethene oxidation by crude extracts of *Methylosinus trichosporium* (cf. M.C. result) and that cyanide

stimulated methanol accumulation from methane. He also found that CO would not inhibit the monooxygenase, which casts doubt on the report<sup>18</sup> that the CO-binding cytochrome C is the immediate electron donor of the hydroxylase. Tonge also found that amytal was a strong inhibitor of the *Methylosinus trichosporium* monooxygenase<sup>24</sup> consistent with the proposed intermediacy of an electron chain in the reduction of the hydroxylase. Stirling<sup>21</sup> was unable to repeat this result and found that amytal had very little effect on the hydroxylation of methane.

It would appear from Stirling's results that the monooxygenase systems from *Methylosinus trichosporium* and M.C. are very similar and they differ from that of *Methylomonas methanica* only in susceptibility to inhibitors and in the particulate nature of the latter. In this respect the latter enzyme appears to be more similar to the monooxygenase from *Methylococcus capsulatus* strain Texas than to that derived from the Bath strain.

#### 1.2.4 Purification and Resolution

The monooxygenase system from M.C. has been separated into three components and partial characterisation of these components has been achieved by a variety of chemical and physical techniques.

Fraction A<sup>25</sup> is a large non-heme iron-containing protein of molecular weight about 200,000. The function of this component is not known with certainty but it could be the hydroxylase. It is necessary for the recombined enzyme complex to function.

Fraction B<sup>25</sup> is a small colourless protein of molecular weight about 15,000. Again its function is unknown, but it may be responsible for the binding of subunits A and C together, because it is not absolutely necessary in the reconstituted enzyme system

but does enhance the efficiency of the system by 2 to 4-fold.

Both of these fractions are comparatively stable at 0°C and no further work on the characterisation of them has been reported.

Fraction C was found to be unstable when stored at 0°C unless a reductant such as sodium thioglycollate, dithiothreitol or NADH were present<sup>25</sup>. Determination of the molecular weight by gel filtration gave a value of 44,600 for fraction C. Gel electrophoresis on S.D.S. polyacrylamide gave the molecule's molecular weight as 39,000, indicating that it is composed of a single polypeptide<sup>26</sup>. Isolated component C gives a yellow solution whose spectrum is shown in Fig. 1.9. Boiling releases an FAD compound (also shown in Fig. 1.9) that was identified by comparison of its absorption spectrum with authentic FAD, by t.l.c. and by its fluorescence excitation and emission spectra (Fig. 1.10)<sup>26</sup>. Colourimetric analysis showed 1 mol of component C to contain 1.00 mol of FAD<sup>25</sup>.

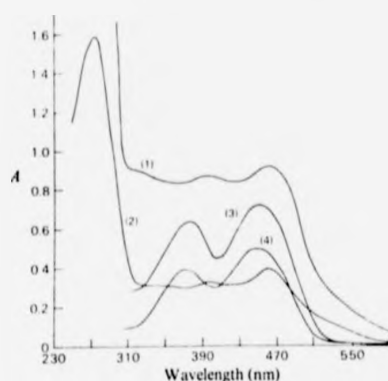


Fig. 1.9 Absorption spectra of purified component C and of its prosthetic group

Component C was purified and prosthetic group prepared from it as described in the Materials and Methods section. Absorption spectra were measured in 20 mM sodium phosphate buffer, pH 7. (1), Purified component C (3.6 mg of protein/ml); (2), purified component C (1.4 mg of protein/ml); (3), prosthetic group (prepared by boiling a solution of component C containing 3.6 mg of protein/ml); (4), FAD (0.045 mM).

Fig. 1.9

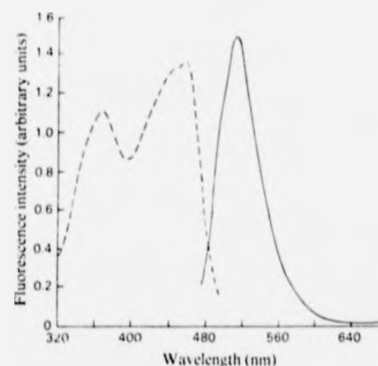


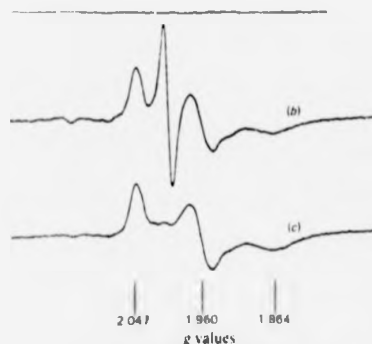
Fig. 1.10 Fluorescence emission and excitation spectra of the prosthetic group

Prosthetic group was prepared from a solution of purified component C (8 mg of protein/ml) as described in the Materials and Methods section. —, Emission spectrum with excitation at 450 nm; ---, excitation spectrum with emission at 520 nm.

Fig. 1.10

Purified component C does not show an ESR signal, but reduction with NADH gave rise to a free radical signal at  $g = 2.002$  attributed to the flavin semiquinone; complete reduction abolishes this signal<sup>26</sup>. The absorption spectrum taken during this titration of component C with NADH shows a progressive decrease in the intensity of the peak at 465 nm. The spectrum of fully reduced C was obtained on the addition of 1.2 mol NADH per mol of enzyme suggesting that component C accepts two electrons per molecule. If this titration was carried out anaerobically a new peak was seen at 570–630 nm, attributed again to the semiquinone<sup>26</sup>.

The ESR spectrum shown in Fig. 1.11 shows additional peaks when compared to that of authentic flavin semiquinone. These peaks are believed to be due to an  $\text{Fe}_2\text{S}_2$  centre. Colourimetric analysis shows one mol of these centres per mol of component C<sup>25</sup>. The structure of this  $\text{Fe}_2\text{S}_2$  centre is believed to be that shown in Fig. 1.12.



*E.p.r. spectra of component C*

Component C (0.07  $\mu\text{mol}$ ; 0.3 ml) in 50 mM-Tris HCl buffer, pH 7, was made anaerobic by repeated evacuation and flushing with argon (a). NADH (5  $\mu\text{mol}$ ) was added anaerobically to the sample and the spectrum recorded after incubation for 1 min at room temperature (b). The fully reduced spectrum (c) was obtained by adding a few crystals of solid sodium dithionite to sample (b) and leaving it at room temperature for 1 min. The spectra were recorded at 19 K at a microwave frequency of 9.232 GHz and modulation of 1.0 mT. The microwave powers were: (a) 10 mW; (b) 1 mW; (c) 1 mW.

Fig. 1.11



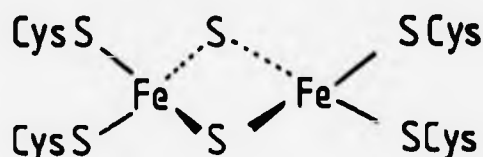


Fig. 1.12

The function of component C is believed to be that of an NADH reductase because purified component C can effect the reduction by NADH of a number of acceptors. Purification of this component can be achieved by binding it to 5'-AMP-Sepharose 4B and eluting with NADH. The apparent  $K_m$  values for NADH and NADPH were found to be 50  $\mu$ M and 15.5 mM, respectively, which supports the belief that NADH is the physiological electron donor<sup>26</sup>.

### 1.3 CYTOCHROME P<sub>450</sub>

Cytochrome P<sub>450</sub> is the name given to a large family of isozymes that have been found in most prokaryotic and eukaryotic forms of life. Its major function in all of these organisms is that of a monooxygenase. Cytochrome P<sub>450</sub> is a misnomer because the cytochrome portion of its name implies that it functions as an electron carrier. Before its physiological function was known, it derived its name because of the similarity of its absorption spectrum to that of the 'b' type cytochromes. This absorption spectrum shows a large Soret band at 450 nm for the reduced CO adduct of P<sub>450</sub> (see Fig. 1.13).

The enzyme was first observed in 1955 by G. R. Williams<sup>27</sup> who was working with rat liver microsomes. Due to its membrane-bound nature and corresponding insolubility no further characterisation

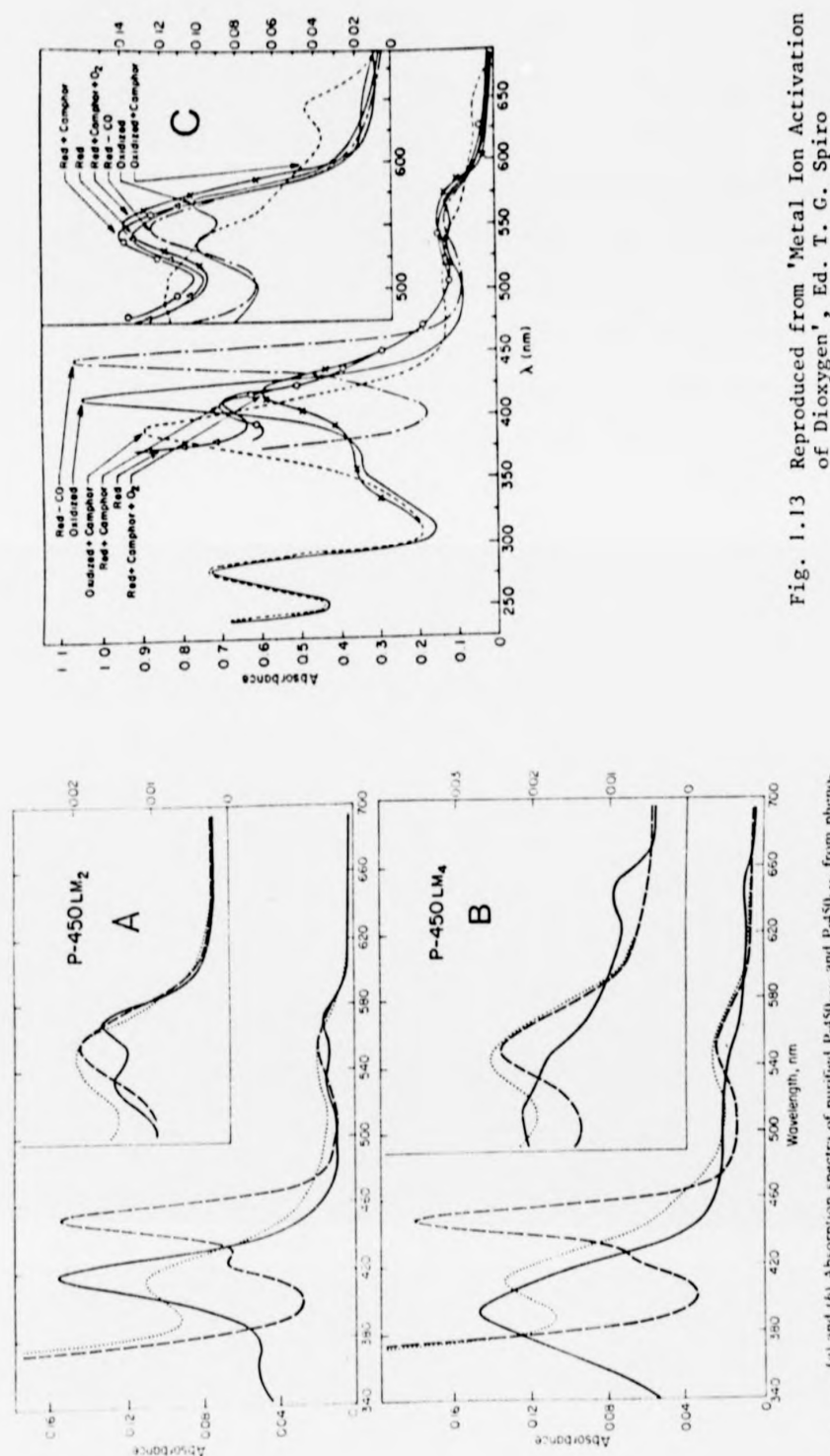


Fig. 1.13 Reproduced from 'Metal Ion Activation of Dioxygen', Ed. T. G. Spiro

(a) and (b) Absorption spectra of purified P-450<sub>LM2</sub> and P-450<sub>LM4</sub> from phenobarbital- and benzoflavone-induced rabbits, respectively, taken from reference 31. (c) Absorption spectra of P-450<sub>LM4</sub> from *Pseudomonas putida*, taken from reference 25. The concentration of the liver cytochromes, based on heme analysis, was 1.40  $\mu$ M for P-450<sub>LM2</sub> and 1.53  $\mu$ M for P-450<sub>LM4</sub> in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol. The spectra were recorded at 20 to 22°C: (—) oxidized; (---) reduced; (· · · ·) reduced CO complex. P-450<sub>LM2</sub> ( $\epsilon_{415} = 87$  at 391 nm for oxidized cytochrome with camphor present) was in 0.05 M potassium phosphate buffer, pH 7.4, containing 0.3 mM D-camphor where

was possible until 1962 when Omura and Sato<sup>27</sup> showed it to be a haemoprotein. One year later, Estabrook<sup>27</sup> and his coworkers showed that its physiological function was that of a monooxygenase responsible for the C-21 hydroxylation of steroids and detoxification of certain drugs. It has since become evident that this detoxification of xenobiotics is a double-edged sword in that the action of P<sub>450</sub> on certain polycyclic aromatic compounds can cause them to become potent carcinogens<sup>28</sup>. Since its discovery P<sub>450</sub> has been found in a wide variety of life forms as table 1.2 shows. This table also demonstrates the remarkable variety of compounds that serve as substrate to this family of enzymes. A list of functions that have been attributed to this enzyme includes aliphatic and aromatic hydroxylation, N-oxidation, sulphoxidation, epoxidation, N, S, and O-dealkylation, peroxidation, deamination, desulphuration and dehalogenation, in addition to the reduction of azo and nitro groups, N-oxides and epoxides<sup>29</sup>.

Major differences exist in the nature of the cytochrome P<sub>450</sub> depending on its source. Normally the enzyme isolated from prokaryotic sources, such as the camphor hydroxylase from *Pseudomonas putida*, is soluble and relies on the activity of three components<sup>27</sup>. The first of these enzymes is an NADPH<sub>2</sub>-linked flavoprotein whose function is the oxidation of the NADPH<sub>2</sub> cofactor. The reducing equivalents obtained by this enzyme are then passed to the second entity in the chain, an iron-sulphur protein. As isolated from *P. putida* this protein contains a two iron, two sulphur cluster and is called putidaredoxin<sup>27</sup>. The major function of this component is that of electron transport to the cytochrome P<sub>450</sub> monooxygenase, where the reducing equivalents are utilised in the hydroxylation reaction.

Cytochrome P<sub>450</sub> isolated from eukaryotic microsomes is,

TABLE 1.2  
Cytochrome P<sub>450</sub> and Biological Hydroxylation Reactions

Cytochrome P<sub>450</sub>-dependent Monooxygenases

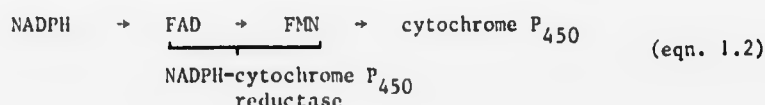
Organism/organ	Subcellular localisation	Substrate	Product	Ref.
<u>Bacteria</u>				
<i>P. putida</i>	Cytosol	Camphor	5-OH-exo-camphor	27
<i>Bacillus magisterium</i>	Cytosol	3-Oxo- $\Delta^4$ steroid	15 $\beta$ -OH-steroids	28
<u>Fungi</u>				
<i>Cunninghamella bairdieri</i>	Microsomes	Xenobiotics	Alcohol, phenols epoxides	29
<i>Rhizopus nigricans</i>	Microsomes	Progesterone	11 $\alpha$ -OH-progesterone	30
<u>Plants</u>				
<i>Vinca rosea</i>	Microsomes	Geraniol	10-OH-geraniol	31
<i>Echinocystis macrocarpa</i> green endosperm	Microsomes	Kaur-16-ene	Kaurenol	23
<u>Yeast</u>				
<i>S. cerevisiae</i>	Microsomes	Xenobiotics	Alcohols, phenols, epoxides	32
<i>Candida tropicalis</i>	Microsomes	Alkanes, Fatty acids	n-Alcohols $\omega$ -OH-fatty acids	21,22

Table 1.2 Continued

Organism/organ	Subcellular localisation	Substrate	Product	Ref.
<u>Insects</u>				
<i>Musca domestica</i>	Microsomes	Xenobiotics	Alcohols, phenols, epoxides	33
<i>Manduca sexta</i>	Mitochondria	$\alpha$ -Ecdysone	$\beta$ -Ecdysone	34
<u>Mammals</u>				
Adrenal gland	Microsomes	Steroids	21-OH-steroids	13
Adrenal gland	Mitochondria	Deoxycorticosterone	Cortisol	35
Liver	Microsomes	Xenobiotics	Alcohols, phenols, epoxides	36, 37
Liver	Mitochondria	Bile acids precursors	27-OH-bile acids	38
Liver	Microsomes	Cholesterol	7 $\alpha$ -OH-precursors	39
Kidney	Microsomes	25-OH-vitamin D <sub>3</sub>	1,25-di-OH-vitamin D <sub>3</sub>	40
Kidney cortex	Microsomes	Fatty acids	$\omega$ -OH fatty acids	41

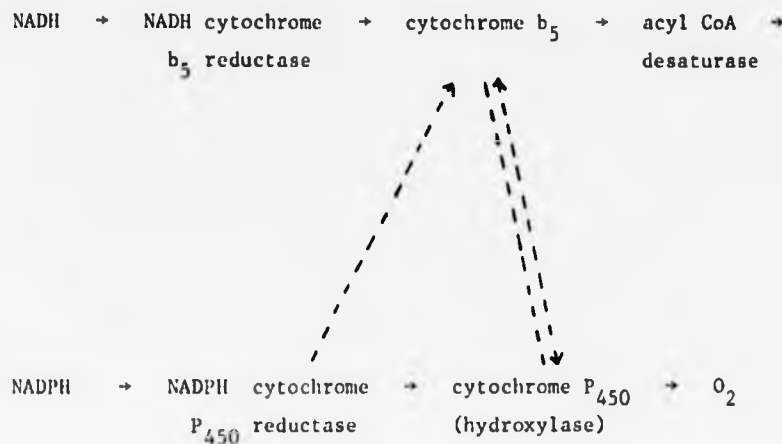
Reproduced from V. Ullrich, Topics in Current Chemistry, 1979, 87

As previously mentioned, a membrane-bound entity that relies for its activity on the presence of two enzymes<sup>27</sup>. As with the enzyme isolated from prokaryotic sources the first of these is an NADPH reductase initially called NADPH-cytochrome C reductase, but now more correctly called NADPH-cytochrome P<sub>450</sub> reductase. This enzyme has a requirement for a phospholipid cofactor and contains both FAD and FMN<sup>30</sup>. There is no involvement of an intermediate electron carrier as there is with the prokaryotic systems. The reducing equivalents are passed directly from the reductase to the mono-oxygenase enzyme *via* the path shown in eqn. 1.2 below<sup>30</sup>.



Some evidence exists supporting a postulate that reducing equivalents can also be supplied by NADH *via* a cytochrome b<sub>5</sub> enzyme, catalysed by an FAD-containing cytochrome b<sub>5</sub> reductase<sup>31</sup>. This may fit into the overall electron transport pathway as shown in Fig. 1.14. It has been found that although the cytochrome b<sub>5</sub> pathway can support cytochrome P<sub>450</sub> catalysed hydroxylations, though with a much lower efficiency than the alternate route, there is a synergistic effect when both pathways are available<sup>31</sup>. Like the prokaryotic sources of cytochrome P<sub>450</sub>, eukaryotic mitochondrial sources also require the intermediacy of an iron-sulphur protein as an electron carrier between the reductase and hydroxylase<sup>29</sup>. This finding is consistent with the belief that mitochondria evolved from bacteria having a symbiotic existence in eukaryotic cells.

The liver is the most important organ for the removal of drugs and other xenobiotics and so it is not surprising to find that it is the major source of cytochrome P<sub>450</sub>. It is an



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Fig. 1.14

induceable enzyme, the structure and substrate specificity of the isozyme induced being dependent on the inducing agent used. The terminology used to identify the isozyme present relies on its relative mobility on SDS gel electrophoresis, and consequently on the enzyme's charge and mass. This is shown as a subscript after the enzyme's name, i.e. cytochrome  $P_{450}$  LM<sub>1</sub> to 7, the LM portion indicating the liver microsomal source of the enzyme. Cytochrome  $P_{450\text{cam}}$  refers to the camphor hydroxylating enzyme isolated from *P. Putida*. There are important differences in the various isozymes insofar as structure and substrate specificity is concerned, although there are some features in common. There is little immunochemical cross-reactivity<sup>32</sup>. Some of the differences that have been identified for three of these isozymes are shown in Table 1.3.

TABLE 1.3  
Properties of Purified Cytochrome P<sub>450</sub>

Property	P <sub>450LM<sub>2</sub></sub>	P <sub>450LM<sub>4</sub></sub>	P <sub>405cam</sub>
Inducing agent	Phenobarbital	5,6-Benzoflavone	Camphor
Minimal molecular weight	49,000	55,000	45,000
Apparent molecular weight	300,000	500,000	45,000
Heme content (per polypeptide chain)	1	1	1
C-Terminal amino acid residue	Arginine	Lysine	Valine
N-Terminal amino acid residue	Methionine	(N-Terminus blocked)	Threonine
Absorption maximum (Soret) without substrate added (nm)	418	394	417
Absorption maximum, CO complex (nm)	451	448	447

Reproduced from 'Metal Ion Activation of Dioxygen', Ed. T. G. Spiro, Wiley Interscience, 1980



### 1.3.1 Structure of cytochrome P<sub>450</sub>

The hepatic microsomal enzyme is membrane bound and so it is not surprising to find that it has a requirement for a phospholipid cofactor<sup>29</sup>. This cofactor is phosphatidylcholine and a ratio of phospholipid to cytochrome P<sub>450</sub> of 20:1 has been found to be the optimum ratio in reconstituted systems. Initial work on the isolation of hepatic cytochrome P<sub>450</sub> from its membranous matrix showed that loss of enzymic activity was denoted by a shift of the absorption band at 450 nm to 420 nm<sup>27</sup>. This was due to denaturation of the enzyme, a problem that was overcome by the use of glycerol or some other polyol to protect the enzyme during the solubilisation procedure<sup>27</sup>. The active site of the purified enzyme has been shown to be a hydrophobic cleft or depression in the enzyme's surface with a heme group at the base of this depression<sup>29</sup>.

The presence of a heme group was demonstrated by Omura and Sato, long before the enzyme was purified, on the basis of its absorption spectrum<sup>27</sup> (see Fig. 1.13). These spectra show a major Soret band at 450 nm, with an  $\alpha$  band absorbing at 558 nm; no  $\beta$  band is seen.

The heme group shown in Fig. 1.15 consists of an iron atom coordinated to the four nitrogens of a protoporphyrin IX moiety which provides the four equatorial ligands to the iron atom. Spectral analyses<sup>33</sup> and model studies<sup>34</sup> support the belief that the fifth, axial ligand is the sulphur of a cysteine residue from the polypeptide chain. The sixth position of this hexacoordinate complex is occupied in the resting state by a water molecule, that is displaced by an oxygen molecule during the catalytic cycle<sup>27</sup>.

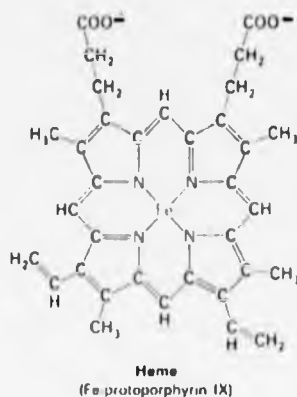


Fig. 1.15

### 1.3.2 Catalytic cycle of cytochrome $P_{450}$

The various steps that are believed to constitute the catalytic cycle of cytochrome  $P_{450}$  are shown in Fig. 1.16.

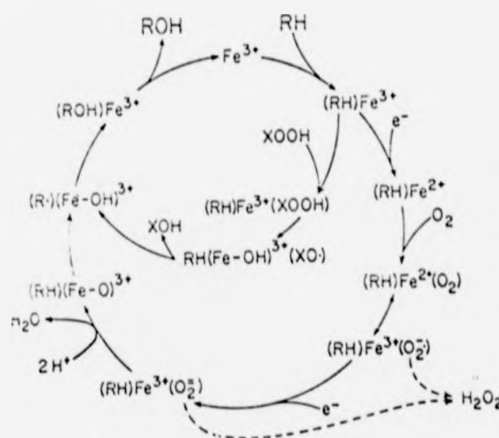
Catalytic cycle of cytochrome  $P_{450}$ 

Fig. 1.16

Spectral analysis has shown that in the resting state cytochrome  $P_{450}$  contains a ferric ion that may be high spin, low spin or a combination of the two depending on the source of the

enzyme<sup>32</sup>. The first step in the catalytic cycle is binding of the substrate with the loss of the water ligand to iron. The change from hexacoordinate to pentacoordinate geometry at iron is accompanied by a change to the high spin state (see Fig. 1.13). As with haemoglobin this change of spin state causes an increase of the ionic radius of the iron, which moves out of the plane of the porphyrin ring<sup>29</sup>.

Stopped flow spectroscopy has shown that the second step of the cycle is a one electron reduction of the ferric-heme complex<sup>29</sup>. Analysis of this step by circular dichroism has shown that a movement of the polypeptide chain around the heme is also associated with this reduction, although the nature and purpose of this movement is unknown.

Binding of the dioxygen molecule is the third step in the sequence. This step can be observed directly by UV/vis spectroscopy with cytochrome P<sub>450cam</sub> and with the adrenal mitochondrial cytochrome P<sub>450</sub>. With cytochrome P<sub>450LM</sub> stopped flow spectroscopy is necessary to detect this very fast reaction.

Step four in the reaction sequence is transfer of a second electron to the ferrous-heme-dioxygen complex. As for dioxygen binding this has not been observed as a discrete step with cytochrome P<sub>450LM2</sub> but it has with P<sub>450cam</sub><sup>29</sup>. In the absence of substrate, addition of the second electron causes the ferrous-heme-dioxygen to break down to the ferric-heme complex and superoxide. Even in the presence of substrate this can occur, possibly to the extent of 50%<sup>29</sup>. There is some experimental support for the belief that *in vivo*, at least for P<sub>450LM</sub> the second reducing equivalent is supplied by the cytochrome b<sub>5</sub> pathway alluded to earlier.

Cleavage of the dioxygen molecule to give water and the active oxygenating species, or some precursor to it, is step five.

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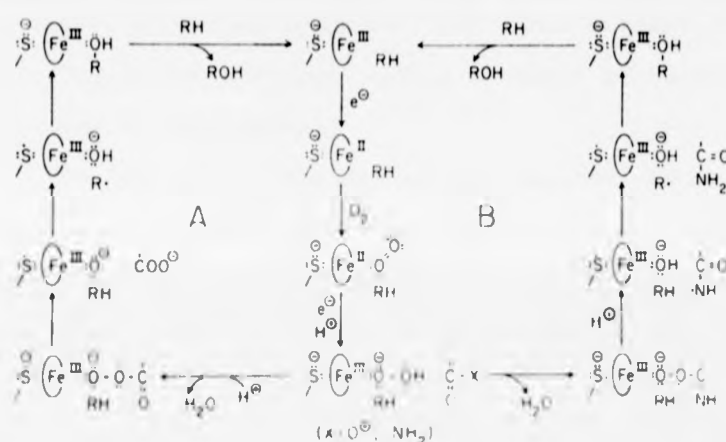
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Cleavage of the dioxygen molecule to give water and the active oxygenating species, or some precursor to it, is step five.

The nature of this step has been the subject of a considerable amount of controversy over the years because the nature of the 'active oxygen' is not known. The mechanism shown in Fig. 1.17 is that given in White and Coons' review<sup>29</sup>. Their use of a peracid or perimidic acid as a precursor to the perferryl complex was originally suggested by Hamilton<sup>1</sup>, but does not claim universal acceptance<sup>35</sup>.

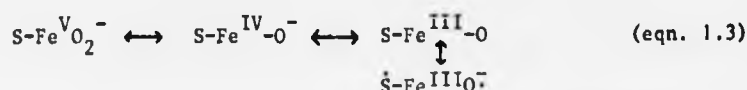


Possible methods of oxygen activation by cytochrome P<sub>450</sub>

Fig. 1.17

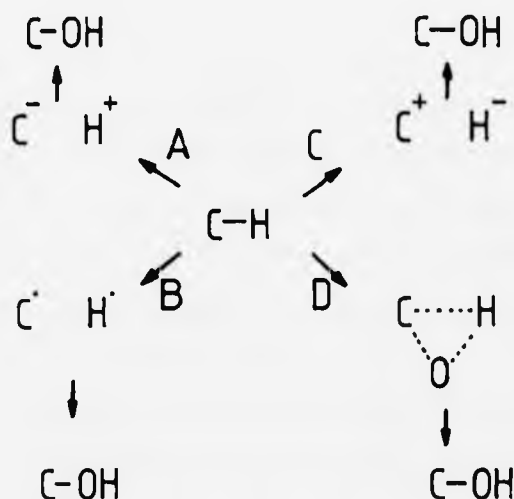
Figure 1.17 shows two pathways: path A proposed heterolytic cleavage of the acylated ferric-heme complex and is called the 'oxenoid' pathway. Path B is termed the quasi-Fenton pathway as it is based on the mechanism of action of the Fenton reagent (see section on models, this Chapter), and proposes a homolytic cleavage of the intermediate perimidic acid. In both of these mechanisms iron is shown in the ferric oxidation state throughout the cycle and serves mainly as a conduit of electrons between its ligands and oxygen. Both mechanisms show an initial hydrogen atom abstraction from substrate to give an alkyl radical which is hydroxylated by  $\text{cys } \text{S}-\text{Fe}^{\text{III}}-\text{OH}$ . The precursor of this iron species in Scheme A is  $\text{cys } \text{S}-\text{Fe}^{\text{III}}-\text{O}^\bullet$  which is a resonance form of the perferryl ion (i.e. Fe at the oxidation level of +5) and has the other resonance forms shown in eqn. 1.3. Additional stabilisation of this species

might be achieved through some involvement of the porphyrin ring system.



Model studies, particularly by Groves<sup>35</sup> (see section on models, this Chapter), suggest that a perferryl species is capable of effecting hydroxylations of hydrocarbons.

The next step in this reaction cycle is product formation, i.e. the insertion of the oxygen atom into the C-H bond of a substrate molecule. There are four methods shown in Fig. 1.18 whereby this may be achieved. The first (path A) involves an initial abstraction



Possible mechanisms of substrate oxidation by monooxygenase enzymes

of a proton by the oxidising agent to give a carbanion, followed by combination of this species with an electrophilic oxygen atom to give the product alcohol. Path B differs in that a hydrogen atom is initially abstracted; in path C a hydride ion is removed. The fourth possibility, path D, involves a concerted, three-centre insertion, which is the type of reaction shown by carbenes and nitrenes that led Hamilton to his suggestion of an 'oxenoid' pathway<sup>1</sup> (see section on models, this Chapter). The fact that an 'NIH' shift (see Chapter 2) is seen in oxidations catalysed by cytochrome P<sub>450</sub> was seen as supporting evidence for the oxenoid pathway. If the mechanism is a concerted insertion (i.e. path D) then hydroxylation reactions should proceed with retention of configuration. This means that in substitutions of a chiral centre the relative configuration of the substituent groups will be retained even though it may change from, e.g. an R to an S configuration. This can occur due to a change in the priorities of the substituents due to the differing priorities of the new group when compared to the replaced one. Furthermore, there should not be a large isotope effect and little selectivity for attack at C-H of a methyl, methylene or methine group. For the other pathways (A-C, Fig. 6) selectivity would be determined by the relative stabilities of the intermediates produced [i.e. carbanion (A), radical (B) or carbocation (C)].

In oxidations of cyclohexene, methylcyclohexane and bicyclo[4,1,0]hexane with cytochrome P<sub>450LM2</sub> the product ratios found were not consistent with a direct insertion<sup>35</sup>. Furthermore, although no overall isotope effects were found, in competition experiments, i.e. those with a mixture of deuterated and non-deuterated substrate, a preference was found for hydrogen abstraction<sup>35</sup>. This evidence, coupled with that obtained from model studies, suggests a radical

mechanism is the one used by this enzyme. Groves found that oxidation of a specifically deuterated norbornane with cytochrome P<sub>450</sub>LM led to some inversion of stereochemistry<sup>35</sup> at the CHD group attacked (i.e. exo D in substrate → endo D in product alcohol). However, Capri<sup>36</sup> has shown that oxidation of a chiral methyl proceeds with retention of configuration.

The final step in the reaction sequence is that of dissociation of product, freeing the enzyme for another catalytic cycle.

The dioxygen molecule that is normally used in the reactions of cytochrome P<sub>450</sub> can be replaced by peroxides and other oxygen donors, e.g. peroxyacids, sodium periodate, iodosylbenzene, cumene hydroperoxide<sup>29</sup>. It has been suggested that such compounds could slot directly into the catalytic cycle and by doing so remove the necessity for both oxygen and the source of the reducing equivalents. Estabrook<sup>37</sup> however, pointed to the different product distribution (e.g. cumene hydroperoxide supported hydroxylation of benz(a)pyrene gave mainly quinones whereas NADPH supported hydroxylation gave phenols) found when cumene hydroperoxide replaced oxygen. White and Coon<sup>29</sup> suggest that the mechanism shown in path B of Fig. 1.17 can explain these distributions because hydrogen abstraction from the substrate is achieved by the alkoxide radical, and not by the ferric-heme-oxy complex. Variations in the nature of this alkoxide radical, they suggest, could account for product distributions observed. Experimental support for their suggestion comes from the trapping of a cumyloxy radical<sup>29</sup> in microsomes exposed to cumene hydroperoxide.



#### 1.4 MODEL SYSTEMS

The main reasons for studying model systems in conjunction with enzymic mechanisms are two-fold. Firstly, in a reaction mixture containing a few known chemical compounds it is much easier to interpret mechanistic results than in a reaction mixture containing complex enzymes and co-enzymes. The second reason for looking at model systems is that an acceptable postulated enzyme mechanism should have an analogy in non-enzymic chemistry.

Care must be taken when extrapolating the results from studies on model systems to an enzymic mechanism because the solvent in the model studies could affect the mechanism. The degree of hydrophobicity of the enzyme's active site could be markedly different from the environment of the model complex. This is seen in the assumption that oxidation of isotopically labelled aromatic compounds giving low or non-existent isotope retention indicates a pathway other than that involving an arene oxide with concomitant NHH shift, because the retention values have been shown to be solvent dependent in both enzymic<sup>38</sup> and model<sup>39</sup> hydroxylations.

Occasionally the most obvious model system may not be the most informative. Such is the case with cytochrome-P<sub>450</sub> where a reasonable model should be an isolated iron-porphyrin complex in the presence of oxygen. Such complexes have been made that are capable of catalysing hydroxylation reactions<sup>34,40</sup>, but these have not, as yet, yielded much information on the mechanism of action, although Sakurai's work<sup>34</sup> has been useful in identifying the axial ligands of the heme group (see section on cytochrome P<sub>450</sub> in this Chapter).

Ideally a good model for a monooxygenase enzyme should be

able to catalyse all of the various reactions that the enzyme itself can perform, i.e. olefin epoxidation, aliphatic and aromatic hydroxylations. Although aromatic hydroxylation and olefin epoxidation are not difficult to achieve, until recently few model systems were able to effect the hydroxylation of unactivated aliphatic hydrocarbons. This was unfortunate because this class of compounds was potentially the most useful for probing the reaction mechanism<sup>35</sup>.

The similarity between many monooxygenase reactions and the reactions of carbenes and nitrenes prompted Hamilton to suggest an 'oxenoid' or oxygen transfer reagent as the active oxygen species. Carbenes and nitrenes both have six electrons in their outer shells and so it was presumed that the active oxygen species would also be electron deficient and would show electrophilic characteristics.

The best understood example of an oxenoid mechanism is the epoxidation of olefins by peracids<sup>41</sup>. This is believed to proceed *via* either the one step reaction mechanism shown in Fig. 1.19 or the cyclic intermediate shown in Fig. 1.20. Trifluoroperacetic acid is the most reactive of the peracids and is capable of hydroxylating aromatic rings. This reaction does proceed with an NIH shift, although the retention of isotopic labels is rather low<sup>42</sup>. These results led Hamilton to suggest that a peracid, or possibly a peramide, might be the active oxygen species in monooxygenase reactions (this mechanism is shown for some copper-containing enzymes in the section on monooxygenases in this Chapter).

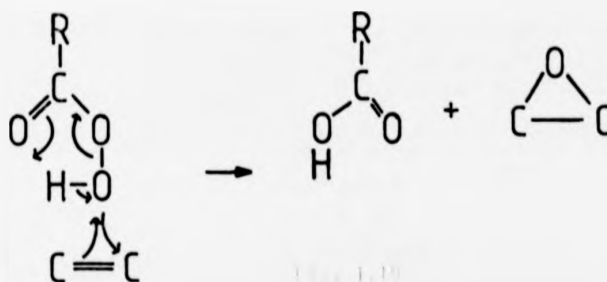


Fig. 1.19

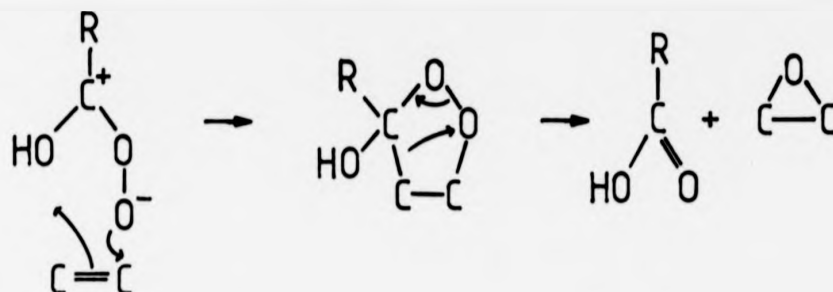
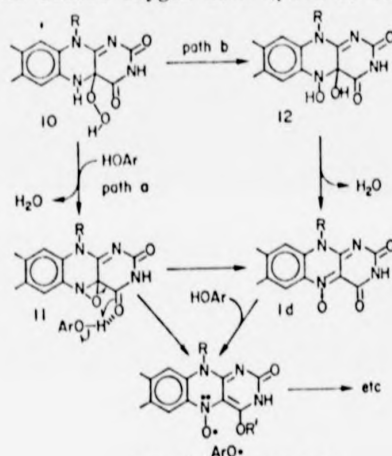


Fig. 1.20

The reactions of the peracids provide model systems that do not contain a metal ion. Other metal-free systems include the aromatic hydroxylations achieved by photolysis of aromatic  $N$ -oxides<sup>43,44</sup>. These reactions exhibit an NIH shift, the quantity of isotope retained being solvent dependent<sup>44</sup>.

Frost and Rastetter<sup>45</sup> believe that this finding is relevant to the mechanism of action of flavoenzymes and have demonstrated that a flavin- $N_5$ -oxide is able to hydroxylate aromatic compounds *via* a radical mechanism. They suggest that the 4a-hydroperoxy intermediate seen in the reaction of the flavoenzymes yields an  $N_5$ -oxide either *via* an oxaziridine (path a) or by electrophilic attack of the lone pair on nitrogen ( $N_5$ ) on the distal oxygen atom (path b) [*cf.* Fig. 1.21].



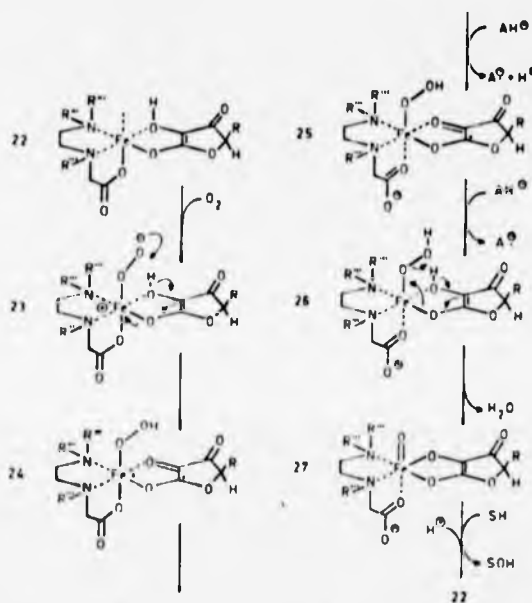
2d, R' = H or electron pair  
Fig. 1.21

Lindsay-Smith *et al.*<sup>46</sup> showed that oxidation of [4-<sup>2</sup>H]phenylalanine with autoxidised 1,3,10-trimethyl-5,10-dihydroalloxazine gave tyrosine with relatively low levels of deuterium retention. This result was concluded to invalidate the pathway postulated by Hamilton (carbonyl-oxide intermediate: see section on monooxygenases in this Chapter) and was believed to support a free radical mechanism for monooxygenases.

Amongst the earliest of the metal-containing model systems for monooxygenase enzymes was that discovered by Udenfriend<sup>47</sup>. It is now called the Udenfriend system and consists of ferrous ions, EDTA, ascorbic acid and oxygen. EDTA is not absolutely necessary, although it does increase the efficiency of the system. Ascorbic acid serves as the reductant and can be replaced by a number of other reductants including tetrahydrobiopterin, N-benzyl-1,4-dihyronicotinamide and diaminopurine<sup>32</sup>. The Udenfriend system is able to catalyse most of the reactions of a typical monooxygenase in that it can epoxidise olefins, and hydroxylate aliphatic and aromatic hydrocarbons. The observed product distribution of its reaction with aromatic compounds suggests an electrophilic oxygenating agent, although no NIH shift<sup>42</sup> occurs. The lack of an NIH shift coupled with the fact that some attack at the 3-position of anisole is seen argues against the intermediacy of an arene oxide in the reaction sequence. Arene oxides are believed to be formed by the attack of a singlet oxenoid species on an aromatic ring.

The hydroxylating agent in the Udenfriend system is not the hydroxyl or superoxide radical, or hydrogen peroxide. Hamilton has suggested that it is a species derived from triplet dioxygen and a metal ion. In this type of complex the unpaired electrons originally on an oxygen atom of dioxygen, could be transferred to the metal, with stabilisation of the complex. Following such

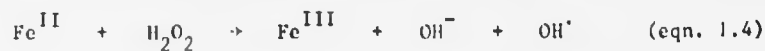
considerations Visser<sup>48</sup> suggested the mechanism shown in Fig. 1.22 for the Udenfriend system.



Proposed reaction sequence for Udenfriend's system. R = -CHOH-CH<sub>2</sub>OH R' = -CH<sub>2</sub>-COO<sup>-</sup>

Fig. 1.22

The major difficulty in determining the mechanism of the Udenfriend system arises because its reactions are not clean, i.e. a complex mixture of products is formed in hydroxylations mediated by this system. This problem does not arise when Fenton's reagent is used. This reagent consists of ferrous ions and hydrogen peroxide, and is able to oxidise most organic compounds. According to the mechanism proposed by Haber and Weiss<sup>32</sup> one electron reduction of hydrogen peroxide by ferrous ions gives hydroxide and an hydroxyl radical (eqn. 1.4) as the hydroxylating agent.



Aromatic hydroxylations using Fenton's reagent did not show an NIH shift<sup>42</sup>. This fact, coupled with the unlikelihood of a hydroxyl radical being the active hydroxylating species in enzymic reactions appeared to detract from the value of this reagent as a monooxygenase model. However, by replacing the aqueous solvent used in the original Fenton's reagent by acetonitrile, Castle *et al.*<sup>49</sup> found some deuterium retention (i.e. NIH shift) in aromatic hydroxylations. Moreover, they showed that the amount of deuterium retained was related to the water content of the solvent<sup>39</sup> (see Table 1.4 below). This suggested that the hydroxylating species was probably radical in character, possibly  $\text{FeO}^{\text{II}}$  or  $\text{FeO}^{\text{III}}$  or maybe even a triplet oxenoid species.

Groves<sup>50</sup> has shown that oxidation of cyclohexanol by Fenton's reagent gives a mixture of products, containing predominantly *cis*-cyclohexane-1,3-diol. Using 7-hydroxynorbornane as substrate he obtained *exo*, *syn*-2,7-dihydroxynorbornane<sup>50</sup>. For both of these hydroxylations he suggested that the hydroxyl group present in the substrate serves to direct the oxidation leading to the observed regio- and stereospecificities. Using deuterium-labelled 7-hydroxynorbornane he demonstrated a 2,6-hydrogen shift in its oxidation and suggested an  $\text{Fe}^{\text{IV}} = \text{O}$  species as the active hydroxylating group. The mechanism proposed<sup>50</sup> for these hydroxylations is shown in Figs. 1.23 and 1.24, and consists of an initial abstraction of a hydrogen atom to give an intermediate radical, which is rapidly oxidised to a carbenium ion by the Fe-OH group. Transfer of the hydroxyl group from the Fe-OH moiety to the carbenium ion then gives the diol product.

Table 1.4

Effect of solvent on the deuterium retention of [4-<sup>2</sup>H]chlorobenzene on oxidation by modified Fenton's reagent

H <sub>2</sub> O content (mmol)	NIH shift (%)
0	38
2.8	37
5.6	37
11.1	34
16.7	29
38.9	15
44.5	10
83.4	9
172	12
284	14
450	16

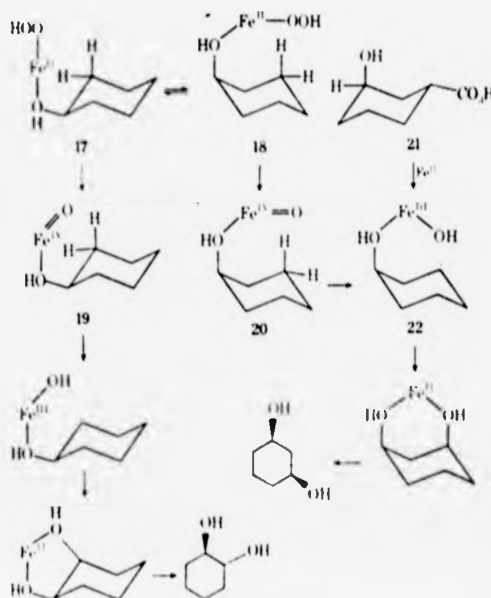


Fig. 1.23

Reproduced from J. T. Groves and M. Van Der Puy,  
*J. Am. Chem. Soc.*, 1976, 98, 5290

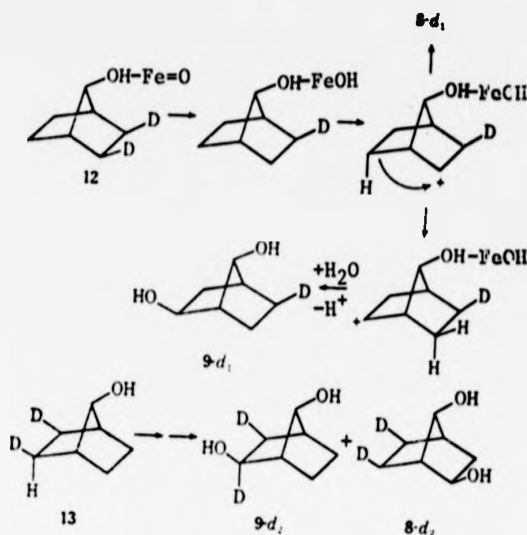


Fig. 1.24

Reproduced from J. T. Groves and M. Van Der Puy  
*J. Am. Chem. Soc.*, 1976, 98, 5290

Other model systems, related to Fenton's reagent or the Udenfriend system, include the so-called Hamilton system. This contains ferric instead of ferrous ions, hydrogen peroxide and a redox active diphenol such as catechol, which complexes to the iron (*cf.* EDTA in the Udenfriend system) and provides the reducing power. The Hamilton system is believed to operate *via* a radical mechanism for the oxidation of both aliphatic and aromatic hydrocarbons, the latter showing little NIH shift.

As with cytochrome P<sub>450</sub>, it is possible to replace dioxygen by a peracid in a modified Fenton's reagent. Groves<sup>51</sup> found that the oxidation of cyclohexanol by ferrous perchlorate and meta-chloroperbenzoic acid in acetonitrile gave, in addition to cyclohexanone, a mixture of all possible diols, although the 1,2-diol was the major one formed. Even though this reaction pathway



is believed to involve radical intermediates the hydroxylation is stereospecific. This means that the developing carbon radical is captured before inversion takes place.

In the majority of the model systems described above the hydroxylating agent is believed to be an  $\text{Fe} = \text{O}$  species, consistent with the proposed 'active oxygen' in the cytochrome  $\text{P}_{450}$  reactions (see section on cytochrome  $\text{P}_{450}$ , this Chapter).

Model systems have been described which generate an active hydroxylating species based on metal ions that are not normally found in monooxygenase enzymes (cobalt, tungsten, vanadium and molybdenum complexes<sup>32,52</sup>). Sharpless has shown that a chromyl complex<sup>53</sup> has aromatic hydroxylating capacity and that this proceeds with an NIH shift.

## CHAPTER I - REFERENCES

1. G. A. Hamilton, 'Molecular mechanisms of oxygen activation', Ed. O. Hayaishi, Academic Press, 1974, and references cited therein.
2. O. Hayaishi, M. Katagin & S. Rothberg, *J. Am. Chem. Soc.*, 1955, 77, 5450
3. H. S. Mason, W. L. Foulkes & E. Peterson, *ibid.*, 1955, 77, 2914
4. R. J. P. Williams, 'Oxygen and Life', Proceedings of the 2nd BOC Priestly Conference, Birmingham, 1980, and references cited therein
5. J. Colby, D. I. Stirling & H. Dalton, *Biochem. J.*, 1977, 165, 395
6. C. Walsh, *Ann. Rev. Biochem.*, 1978, 44, 881, and references cited therein
7. C. Walsh, 'Enzymatic Reaction Mechanisms', W. H. Freeman and Co., 1979, and references cited therein
8. C. Walsh, *Acc. Chem. Res.*, 1980, 13, 148, and references cited therein
9. P. Hemmerich, Progress in the Chemistry of Organic Natural Products, 1976, 33, 451, Ed. H. Grisbach, Springer-Verlag, Berlin
10. T. Ljones & T. Skotland, *F.E.B.S. Lett.*, 1979, 108, 25
11. T. Ljones, *et al.*, *F.E.B.S. Lett.*, 1978, 92, 81
12. S. W. May, R. S. Phillips, P. W. Mueller & H. H. Herman, *J. Biol. Chem.*, 1981, 256, 8470
13. R. Whittenbury, K. C. Phillips & J. F. Wilkinson, *J. Gen. Microbiol.*, 1970, 61, 205
14. J. Colby, H. Dalton & R. Whittenbury, *Ann. Rev. Microbiol.*, 1979, 33, 481, and references cited therein
15. D. W. Ribbons, *J. Bacteriol.*, 1975, 122, 1351
16. J. Colby, H. Dalton & R. Whittenbury, *Biochem. J.*, 1975, 151, 459
17. T. Ferenci, T. Strom & J. R. Quayle, *J. Gen. Microbiol.*, 1975, 91, 79

18. G. M. Tonge, D. E. F. Harrison & J. J. Higgins, *Biochem. J.*, 1977, 161, 333
19. D. I. Stirling, J. Colby & H. Dalton, *Biochem. J.*, 1979, 177, 361
20. S. W. May, S. L. Gordon & M. S. Steltenkamp, *J. Am. Chem. Soc.*, 1977, 99, 2017
21. D. I. Stirling & H. Dalton, *Eur. J. Biochem.*, 1979, 96, 205
22. J. Colby & H. Dalton, *Biochem. J.*, 1976, 157, 495
23. Meeting of the 3rd Int. C. Symposium, University of Sheffield, 1980
24. G. M. Tonge, D. E. F. Harrison, C. J. Knowles & I. J. Higgins, *F.E.B.S. Lett.*, 1975, 58, 293
25. J. Colby & H. Dalton, *Biochem. J.*, 1979, 177, 903
26. J. Colby & H. Dalton, *Biochem. J.*, 1978, 171, 461
27. R. Sato & T. Omura, 'Cytochrome P<sub>450</sub>', Academic Press, 1978, and references cited therein
28. D. M. Jerina & J. W. Daly, *Science (Washington, D.C.)*, 1974, 185, 573
29. R. E. White & H. J. Coon, *Ann. Rev. Biochem.*, 1980, 315, and references cited therein
30. J. L. Vermilion, D. P. Ballou, V. Massey & M. J. Coon, *J. Biol. Chem.*, 1981, 256, 266
31. A. J. Paine, 'Essays in Biochem.', 1981, 17, 85, Ed. P. N. Campbell & R. D. Marshall, and references cited therein
32. 'Metal ion Activation of Dioxygen', Ed. T. G. Spiro, Wiley Int., 1980, and references cited therein
33. V. Ullrich, *Topics in Current Chemistry*, 1979, 87, Ed. F. L. Boschke, Springer-Verlag
34. H. Sakurai, S. Shimomura & K. Ishizu, *Biochem. Biophys. Res. Commun.*, 1981, 101, 1102
35. J. T. Groves, 'Advances in Inorganic Biochem.', Ed. G. L. Eichhorn & L. G. Marzilli, Elsevier, 1979, and references cited therein
36. E. Caspi, S. Shapiro & J. Piper, *J. Chem. Soc. Chem. Comm.*, 1981, 1196
37. J. Capdevila, R. W. Estabrook & R. A. Prough, *Arch. Biochem. Biophys.*, 1980, 200, 186

38. J. W. Daly, D. M. Jerina & B. Witkop, *Experientia*, 1972, 28, 1129 and references cited therein
39. L. Castle & J. R. Lindsay-Smith, *J. Mol. Catal.*, 1980, 7, 235
40. T. Shimidzu, T. Iyoda & M. Kanda, *J. Chem. Soc. Chem. Comm.*, 1981, 1206
41. J. March, 'Advanced Organic Chemistry', 2nd Ed., McGraw-Hill, 1977, and references cited therein
42. D. Jerina, *et al.*, *J. Am. Chem. Soc.*, 1967, 89, 3347
43. N. M. Akhtar, *et al.*, *J. Chem. Soc. Perkin Trans. I*, 1980, 1693
44. J. Ogawa, S. Iwasaki & S. Okuda, *Tetrahedron Lett.*, 1981, 22, 3637
45. J. W. Frost & W. H. Rastetter, *J. Am. Chem. Soc.*, 1981, 103, 5242
46. J. R. Lindsay-Smith, *et al.*, *J. Chem. Soc. Chem. Comm.*, 1975, 881
47. S. Undenfriend, C. T. Clark, J. Axelrod & B. B. Brodie, *J. Biol. Chem.*, 1954, 208, 731
48. C. M. Visser, *Bioorg. Chem.*, 1980, 9, 261
49. L. Castle & J. R. Lindsay-Smith, *J. Chem. Soc. Chem. Comm.*, 1978, 704
50. J. T. Groves & M. Van Der Puy, *J. Am. Chem. Soc.*, 1976, 98, 5290
51. J. T. Groves & G. A. McClusky, *J. Am. Chem. Soc.*, 1976, 98, 859
52. A. Nishinaga & H. Tomita, *J. Mol. Catal.*, 1980, 7, 179
53. K. B. Sharpless & T. C. Flood, *J. Am. Chem. Soc.*, 1971, 93, 2316

## CHAPTER 2

### CYCLOPROPANES

#### 2.1 OBJECTIVES

Numerous rearrangements proceeding *via* radical or cationic intermediates are known in the chemistry of cyclopropanes<sup>1</sup>. This makes cyclopropanes potentially valuable substrates for enzymes, because such reactive intermediates might arise in the enzyme-catalysed reactions. Analysis of the products from such reactions might enable certain conclusions to be drawn about their pathways and mechanisms.

#### 2.2 CYCLOPROPANE STRUCTURE

In 1885 Adolf von Baeyer put forward a theory to account for some of the unusual chemistry of small alicyclic compounds<sup>1</sup>. His theory centred around the strain that is present when an  $sp^3$  hybridised carbon has its bond-angle compressed from the tetrahedral value of  $109.5^\circ$  to that of  $60^\circ$  in cyclopropane and  $90^\circ$  in cyclobutane. This angle strain, particularly in the cyclopropanes, leads to a certain degree of instability and consequent increased reactivity. Measurements of heats of combustion show cyclopropane to be  $38.5 \text{ kJ mol}^{-1}$  per methylene group less stable than cyclohexane, the least strained of the small to medium rings<sup>2</sup>.

Quantum mechanical calculations by C. A. Coulson and W. A. Moffit led them to suggest the presence of 'bent' or 'banana' bonds in cyclopropanes (*cf.* Fig. 2.1)<sup>2</sup>. The carbon-carbon bond-length

in cyclopropane of  $1.51 \text{ \AA}$  is in-between that of a typical  $sp^2-sp^2$  C-C single bond ( $1.48 \text{ \AA}$ ) and an  $sp^3-sp^3$  C-C single bond ( $1.53 \text{ \AA}$ ). The hybridisation of the orbitals of the C-C bonds in cyclopropane shows about 17% s character and has been calculated as  $sp^{4.12}$ , whereas the orbitals used in C-H bonding are  $sp^2$  having about 33% s character. Owing to the bent nature of the C-C bonds and their diminished s character, they cannot strictly be called  $\sigma$  bonds.



Fig. 2.1

## 2.3

CHEMISTRY OF CYCLOPROPANES

There are two general types of reaction that cyclopropanes undergo: free radical substitutions and electrophilic additions. In the following discussion, intermediates will be represented by solid lines and a more detailed discussion of their structure will be undertaken in a later section.

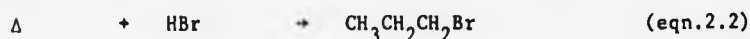
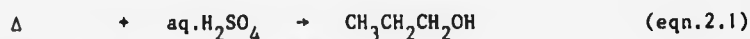
## (a)

Free Radical Reactions

In common with other saturated hydrocarbons, cyclopropanes can undergo substitution *via* a free radical mechanism. In the case of chlorination, under mild conditions, the products are chlorocyclopropane and 1,1-dichlorocyclopropane<sup>3</sup>.

(b) Addition Reactions

The addition of electrophiles to cyclopropanes to give ring-opened products, *cf.* eqns. 2.1 and 2.2 below, lends support to the idea of increased p character in the C-C bonds.



The direction of addition for substituted cyclopropanes obeys the Markownikov rule. Thus, addition of HCl to methylcyclopropane gave only 2-chlorobutane, no 1-chloro-2-methylpropane being found<sup>4</sup>.

As with most other organic molecules, the reaction of cyclopropane with molecular oxygen is extremely slow, but with  $\text{O}(^3\text{P})$  (i.e. ground state atomic oxygen) reaction is very much quicker<sup>5</sup>. Normally the reaction of  $\text{O}(^3\text{P})$  with saturated alkanes proceeds *via* an initial abstraction of a hydrogen atom from the alkane by the oxygen atom. With unsaturated hydrocarbons, however, the reaction differs in that the oxygen atom adds to the compound to produce a short-lived, energy rich compound. This species then undergoes a unimolecular fragmentation reaction<sup>5a</sup>. Earlier in this Chapter it was shown that cyclopropanes can undergo two different types of reaction. These were a hydrogen atom abstraction (*cf.* most aliphatic compounds) and an addition reaction, supporting the hypothesis for extra p-character in the C-C bonds. It is this latter type of reaction that Seala and Wu believe to take place between  $\text{O}(^3\text{P})$  and cyclopropane<sup>5</sup>. They base their argument on the

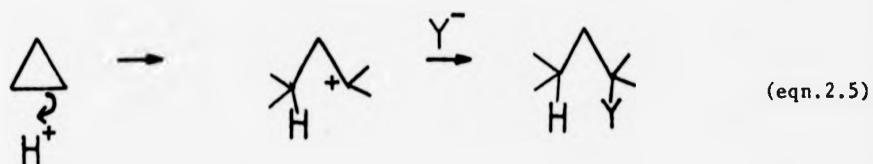
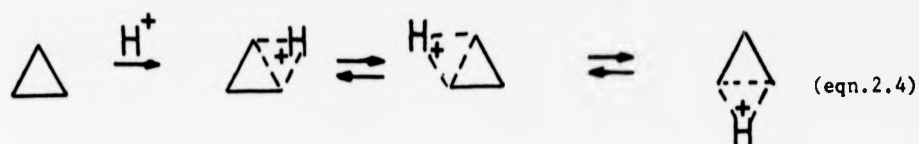
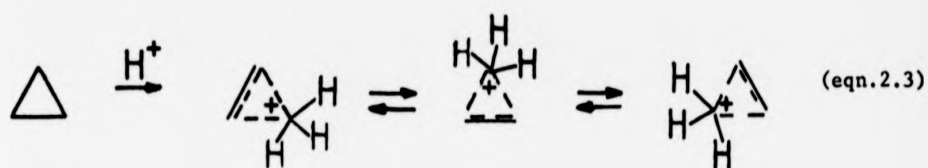
observed product distribution, and the fact that the reaction is slower than the corresponding one with other cycloalkanes, which is a consequence of the unfavourable entropy for attack at the edge of the cyclopropane ring. This is supported by the finding that the pre-exponential factor for the above reaction is very much smaller than for compounds known to react *via* a radical abstraction mechanism<sup>6</sup>. Huie and Herron<sup>6a</sup>, however, still feel that the reaction proceeds *via* an initial hydrogen abstraction step, followed by a rapid combination of the alkyl radical with the oxygen species to generate a 'hot' cycloalkocyl radical, which decomposes to give the observed products.

## 2.4 POTENTIAL INTERMEDIATES IN OXYGENATION PATHWAYS

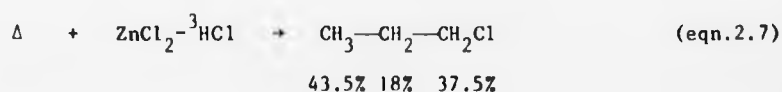
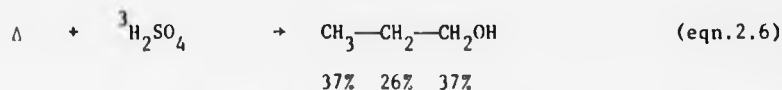
### 2.4(a) CATIONIC SPECIES

The reactions of cyclopropane rings with electrophiles to give ring-opened, substituted propanes go by way of carbenium ions, the exact nature of which is unknown. There have been a number of possibilities put forward of which (a) the corner protonated and (b) the edge-protonated species, shown in equations 2.3 and 2.4, have found most support<sup>1</sup>. Alternatively, electrophilic attack could lead directly to a classical carbenium ion that is captured by a nucleophile as shown in equation 2.5.





There are a few experimental results that eliminate the possibility of some of the proposed cationic species acting as intermediates. Treatment of cyclopropane with tritiated  $\text{H}_2\text{SO}_4$ <sup>7</sup> or Lucas reagent<sup>8</sup> ( $\text{ZnCl}_2$ - $^3\text{HCl}$ ) gave only the 1-substituted propanes with the label distribution shown in equations 2.6 and 2.7 below.



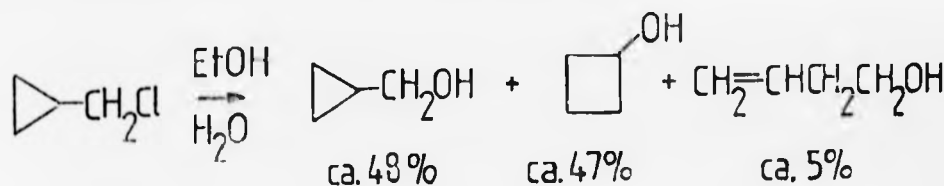
(atom % tritiation shown)

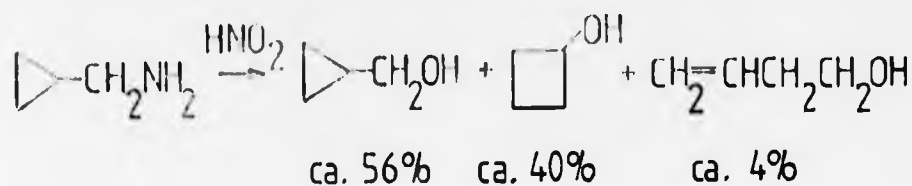
Although there is some difference in the absolute quantities both examples show label in the C2 position, which is not consistent with a non-delocalised classical carbenium ion, i.e. equation 2.5 alone cannot explain these results.

The reaction of HBr with bromocyclopropane showed a mixture of all three dibromopropanes as products. If, as has been stated, the addition obeys the Markownikov rule, then the classical carbenium ion (cf. eqn. 2.5) should give rise to only 1,1-dibromopropane. These results are more consistent with the pathways of equations 2.3 and 2.4, or possibly even a rapidly equilibrating mixture of the two cationic species of these pathways.

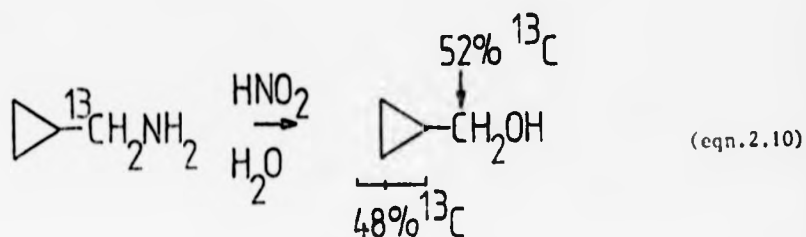
Scala and Wu<sup>5</sup> have pointed out that the reaction of cyclopropane with oxygen is very slow in comparison with other cycloalkanes. This, they argue, may be due to the unfavourable entropy of activation which could be a result of the requirement for attack at the edge of the ring.

The position with regard to the cationic intermediates generated in the solvolysis of appropriately substituted methylcyclopropanes or in the deamination of cyclopropylmethylamine caused by nitrous acid is more complicated. Both of these reactions generate a mixture of products (eqns. 2.8 & 2.9) that the postulated intermediates have to be able to account for<sup>9</sup>. One also has to explain the scrambling of label that is shown in equation 2.10 below, and the unusual, high solvolysis rates found in these systems.

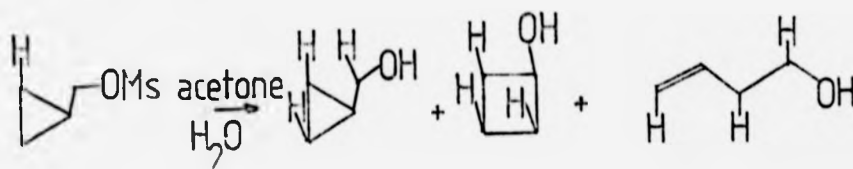




(eqn. 2.9)



The results of the hydrolysis of cyclopropylcarbinyl-0-methanesulphonate, completely deuterated except at the position shown in equation 2.11, to give products with hydrogen appearing only at the indicated positions, complement those of equation 2.10.



(eqn. 2.11)

A number of possibilities have been advanced for the structure of the intermediate involved in the reactions of the above type (eqns. 2.8-2.11) and these are shown in Figs. 2.2-2.5.



Fig. 2.2 The 'unsymmetrical homoallylic' ion.

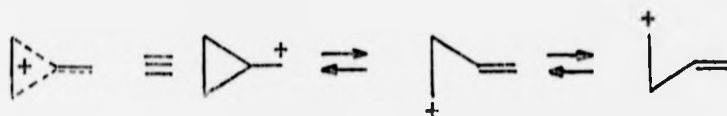


Fig. 2.3 The 'symmetrical homoallylic' ion.

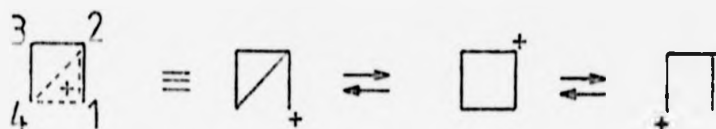


Fig. 2.4 The bicyclobutonium ion

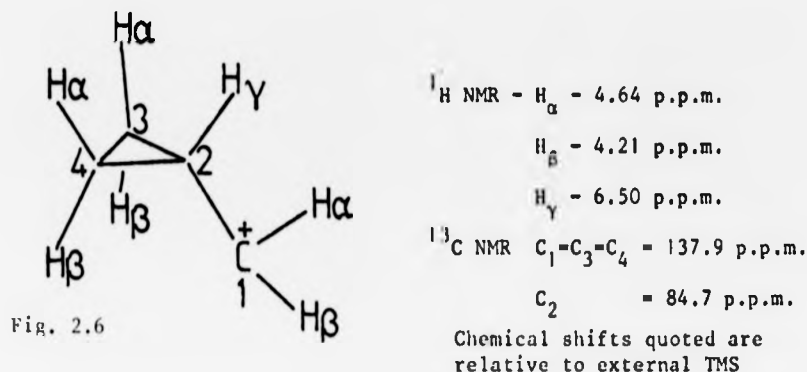


Fig. 2.5 The tricyclobutonium ion.

The tricyclobutonium ion (Fig. 2.5) has been discounted both on the grounds of theoretical calculations and because the methylene groups are not always completely equilibrated during some reactions<sup>9</sup>.

Intermediacy of the tricyclobutonium ion must equilibrate all three methylene groups.

The work of Schleyer and Van Dine on the effects of methyl substitution at the  $\beta$ -carbons on the rates of solvolyses of cyclopropylcarbinyl substrates indicates that the two ring methylene carbons of the parent system are equivalent<sup>9</sup>. This conclusion is supported by the work of Olah and his co-workers on the NMR spectra of cyclopropylcarbinol in  $\text{SbF}_5\text{-SO}_2\text{ClF}$  at  $-70^\circ\text{C}$  that gives the  $^{13}\text{C}$  and  $^1\text{H}$  resonances shown in Fig. 2.6



Both of the  $\beta$ -methylene carbons are equivalent so the 'unsymmetrical homoallylic' ion (Fig. 2.3) is unlikely to be the structure of the intermediate. This NMR result also argues against the possibility that the increased solvolysis rate of cyclopropylcarbinyl chloride relative to that of other appropriately substituted cycloalkanes is simply due to an inductive effect stabilising the carbenium ion. It is doubtful that an inductive effect could lead to such a large downfield shift of the C3/C4 protons. This is more likely to have arisen from conjugation leading to charge delocalisation over the entire ring with consequent stabilisation of the carbenium ion. This stabilisation is maximised in the 'symmetrical homoallylic' ion in Fig. 2.3. Fig. 2.7 shows the conformation of this structure that allows maximum overlap of the empty p orbital with the ring carbon-carbon bonds.

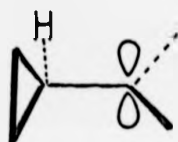


Fig. 2.7

In some polycyclic systems the 'bisected structure' cannot be achieved because of some constraint of the molecular framework.

Thus, the cyclopropyl derivative of 1-chloroadamantane (Fig. 2.8) undergoes solvolysis in 50% ethanol  $10^{-3}$  slower than 1-chloroadamantane (Fig. 2.9), because the cyclopropylcarbinyl cation derived from the cyclopropyl-substituted 1-chloroadamantane possesses a relatively unfavourable perpendicular conformation<sup>9</sup> (Fig. 2.10).



Fig. 2.8



Fig. 2.9

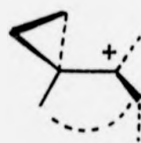


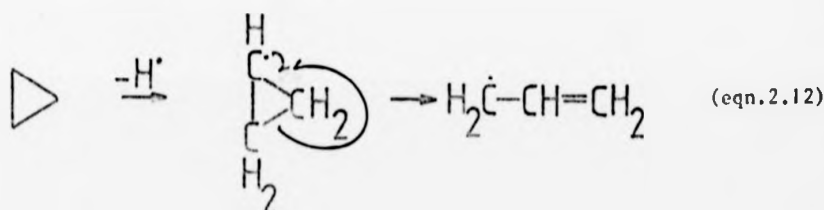
Fig. 2.10

The possibility that the bicyclobutonium ion (Fig. 2.4) is an intermediate in the solvolysis of appropriately substituted methylocyclopropanes receives most support from theoretical studies<sup>10</sup>, but there is no NMR evidence for this species.

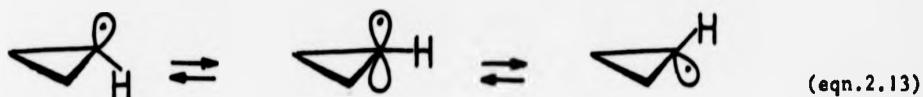
The majority of evidence points towards the bisected structure (Fig. 2.7), which has a barrier to rotation around the Cl-C2 bond of  $107.6 \text{ kJ mol}^{-1}$ <sup>10</sup>.

2.4(b) RADICAL SPECIES

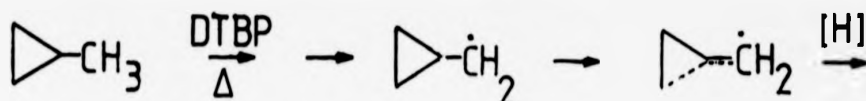
Free radical chlorination of cyclopropane gives products of substitution in which the three-membered ring is retained (*cf.* Section 2(ii)). Due to the amount of ring strain in cyclopropanes, the electrocyclic ring-opening shown in equation 2.12 might be expected to be favourable. However, this does not occur readily because of the high activation energy barrier for both conrotatory and disrotatory ring-opening<sup>11,12</sup>.



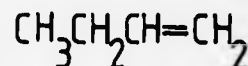
ESR studies on the cyclopropyl radical<sup>13</sup> show the coupling to the  $\alpha$ -hydrogen to be 6.5 G, whereas in planar alkyl radicals, this is found to be approximately 20 G. This low value for the cyclopropyl radical has been accounted for by postulating a pyramidal structure (*cf.* eqn. 2.13). This is supported by the finding that some substituted cyclopropyl radicals retain their configurational integrity during a reaction. However, the parent cyclopropyl radical is presumably rapidly inverting because Kobayashi<sup>14</sup>, who trapped with bromine a deuterated cyclopropyl radical, formed *via* a Hunsdiecker reaction, found the ratio of *cis* to *trans* product to be unity. In agreement, the ESR spectrum<sup>13</sup> shows the  $\beta$ -hydrogens to be equivalent. The inversion of the cyclopropyl radical is believed to proceed *via* a  $\pi$  radical transition state (eqn. 2.13). This inversion was noted by Fessenden and Schuler<sup>13</sup> to be rapid at  $-120^\circ\text{C}$ , and was quoted by Walborsky<sup>15</sup> as having a rate constant of approximately  $10^8 \text{ s}^{-1}$ . Unfortunately, he did not give the temperature at which this was measured.



The activation barrier that prevents the cyclopropyl radical from ring-opening is not present in the cyclopropylcarbinyl radical, because the latter rearranges to the allylcarbinyl radical (*cf.* eqn. 2.14) with a rate constant of  $1.3 \times 10^8 \text{ s}^{-1}$  at  $25^\circ\text{C}$ <sup>16</sup>.



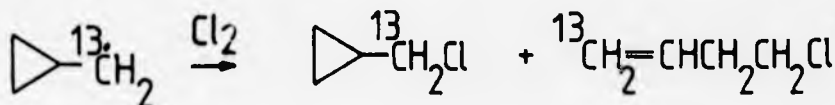
(DTBP = di-*t*-butylperoxide)



(eqn. 2.14)

ESR studies of the cyclopropylcarbinyl radical<sup>17,18</sup> show that it is stable below  $-150^\circ\text{C}$ , but above  $-100^\circ\text{C}$  only the ring-opened allylcarbinyl radical is present. At intermediate temperatures, a mixture of the two radicals is seen. Production of the allylcarbinyl radical from an allylcarbinyl precursor shows no contamination from cyclopropylcarbinyl radical and at temperatures below  $0^\circ\text{C}$  there is no detectable interconversion with the cyclopropylcarbinyl radical.

ESR spectroscopy shows that the C-1 methylene group in the cyclopropylcarbinyl radical does not equilibrate with the C-3/C-4 methylene groups<sup>17</sup>, and that any exchange process similar to that found in the cation is slow on an ESR scale. This is supported by Renk's work, which shows no scrambling of the  $^{13}\text{C}$  label (*cf.* eqn. 2.15)<sup>19</sup>.



(eqn. 2.15)



The lack of any cyclobutyl products from reactions involving cyclopropylcarbinyl radicals argues against a bicyclobutyl radical intermediate. Kochi<sup>17</sup> believes that the low H-2 to C-1 coupling constant is evidence for a bisected structure for the cyclopropylcarbinyl radical (*cf.* the cationic case).

Another possible structure for the cyclopropylcarbinyl radical is shown in Fig. 2.11, and is similar to the 'unsymmetrical cation' structure proposed for the cyclopropylcarbinyl cation (*cf.* Fig. 2.2). This could explain the finding of Hart and Cipriani<sup>20</sup> that the production of the cyclopropylcarbinyl radical from the corresponding diacyl peroxide is faster than for other cycloalkylcarbinyl radicals. However, this may be due to the stabilisation gained from partial opening of the strained 3-membered ring.

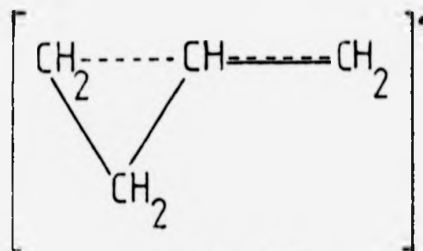


Fig. 2.11

#### 2.4(c) ANIONIC SPECIES

In comparison with the cationic and radical intermediates discussed above, there is very little published work on the unsubstituted cyclopropyl anion. An anionic centre is stabilised on a cyclopropane ring<sup>21</sup>. Like the cyclopropyl radical the cyclopropane anion is not planar. It has been found that the presence of unsaturated substituents adjacent to the anionic centre can, by delocalisation, lower the energy barrier to planarity. This energy barrier has been demonstrated by the

finding that  $^2\text{H}$  exchange of 2,2-diphenylcyclopropyl anion is  $8 \times 10^3$  faster than racemisation<sup>22</sup>. Using optically active 1-bromo-1-methyl-2,2-diphenylcyclopropane, Walborsky *et al.*<sup>23</sup> showed that configurational stability and optical activity were completely retained in a reaction proceeding *via* the anion. This has also been supported by other work<sup>24</sup>.

## 2.5 ENZYMIC OXIDATIONS OF CYCLOPROPANES

Cyclopropane and certain of its derivatives may be instructive substrates in enzymic oxidations, because of the possibility that cyclopropyl intermediates will undergo molecular rearrangements. As is mentioned in Chapters 1 and 3 there is some support for the belief that the enzymic oxidising agent is an electrophile. If this species produced, e.g. a cyclopropyl cation, this could ring-open to an allyl cation. There is some support for the intermediacy of organic radicals in P-450-dependent enzymic processes and this led us to consider the consequences of such intermediates in oxidations of cyclopropanes effected by the M.C. enzyme.

Ring-opening of cyclopropane by an enzymic oxidising agent has been supposedly demonstrated by Ooyama and Foster<sup>25</sup>, working with *Microbacterium vaccae* (JOB 5). Their report that the product of this reaction was propionaldehyde is in some doubt, because the work-up procedure involved an atmospheric distillation followed by addition of acidic 2,4-dinitrophenylhydrazine. This procedure would most likely convert any cyclopropanol formed into propionaldehyde<sup>26</sup>.

*Pseudomonas aeruginosa* has been shown to have a paraffin-oxidising system that can be induced by cyclopropane, but not by methylcyclopropane. These compounds are not, however, oxidised by this system<sup>27</sup>.

With the M.C. enzyme we found that the product of cyclopropane oxidation is cyclopropanol<sup>28</sup>. This was identified by its co-chromatography on GLC with authentic cyclopropanol. No propionaldehyde or allyl alcohol peak could be seen. There was insufficient product available to characterise it spectroscopically, so further characterisation was carried out by treatment with aqueous acid to give propionaldehyde which was isolated as its 2,4-dinitrophenylhydrazone. TLC analysis of the crude DNP showed two spots, one that co-chromatographed with authentic propionaldehyde 2,4-dinitrophenylhydrazone and another that co-chromatographed with acetone 2,4-dinitrophenylhydrazone (from trace acetone contamination of the water used). The derivatisation step required heating to 60°C for 45 mins to convert the cyclopropanol to the propionaldehyde derivative. From an unheated control experiment only one spot was observed corresponding to acetone DNP.

The product peak seen on GLC and ascribed to cyclopropanol was absent when the oxidation was carried out in the presence of acetylene (a known inhibitor of the M.C. enzyme), but was observed when cyanide was present in the reaction mixture. (N.B. Cyanide does not inhibit the M.C. enzyme.)

The fact that no ring-opened product (i.e. allyl alcohol) was observed indicates that a cyclopropyl cation is not an intermediate in the oxidation pathway from cyclopropane to cyclopropanol.

Analysis of the product of oxidation of methylcyclopropane by the M.C. enzyme system using two different GLC columns showed only a single product peak. This peak co-chromatographed on both columns with authentic cyclopropylcarbinol and no peak corresponding to but-3-en-1-ol could be seen. This product peak was not seen in oxidations carried out in the presence of acetylene, but it was present when cyanide

was included in the oxidation medium. Derivatisation of the product of enzymic oxidation of methylcyclopropane with  $\alpha$ -naphthylisocyanate gave a compound that co-chromatographed on t.l.c. with an authentic sample of the  $\alpha$ -naphthylurethane of cyclopropylcarbinol. The 400 MHz  $^1\text{H}$  NMR spectrum of this derivative also supported the conclusion that it was the  $\alpha$ -naphthylurethane of cyclopropylcarbinol. Although impurities in the compound masked the methine resonance at 1.23 p.p.m., the peaks at 0.36 and 0.63 p.p.m. clearly show that the three membered ring is still intact. These resonances appear in the spectrum of the authentic  $\alpha$ -naphthylurethane of cyclopropylcarbinol and are assigned to the pairs of equivalent protons at C-3 and C-4. The protons from the  $\text{CH}_2\text{O}$  group show as a doublet at 4.07 p.p.m. with a coupling constant to the methine hydrogen of ca. 9 Hz. Except for the previously mentioned impurities the spectrum is otherwise the same as that taken of an authentic sample of the  $\alpha$ -naphthylurethane of cyclopropylcarbinol.

Electron impact mass spectral analysis of the  $\alpha$ -naphthylurethane derivative of the product of M.C. oxidation of methylcyclopropane showed an ion at 241 due to the molecular ion (cf. Fig. 2.12). Although the majority of the spectrum is dominated by the uninformative ions produced from the derivative portion of the molecule, there are also peaks at  $m/z$  of 115 and 55 from both the authentic compound and enzymic product. These peaks could be due to the fragments shown in Fig. 2.12.

The evidence presented in the above discussion shows that the only detectable products from the oxidation of cyclopropane and methylcyclopropane, by the M.C. enzyme system, are cyclopropanol and cyclopropylcarbinol, respectively. In neither case could any ring-opened products be seen, arguing against a pathway that involves either a cationic or a radical intermediate in M.C. mediated oxidations.

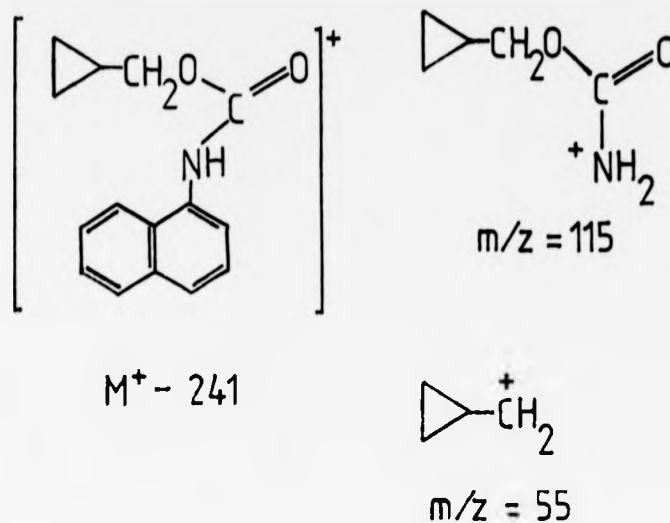


Fig. 2.12

Attention was now focussed on the P-450 dependent mono-oxygenase systems for which there was some evidence of an organic radical being an intermediate in the oxidation pathway. If these systems were to effect the conversion of methylcyclopropane to but-3-en-1-ol, not only would it support the presence of a radical pathway in P-450 dependent enzymes, but it would also reinforce the belief that a similar pathway was not used by the M.C. system. A number of oxidations were attempted with aroclor-induced rat liver microsomes, but in no case was a definite product seen on GLC even though the microsomal system oxidised cyclohexane very efficiently. This could mean either that these cyclopropanes are not substrates for the particular P-450 isozymes present in these particular microsomes, or that the products are further metabolised and consequently not detected.

A number of attempts were made to oxidise methylene-

cyclopropane with the M.C. enzyme system and on one occasion this appeared to be successful, giving a product that had a GLC retention time similar to that of butan-1-ol. Authentic 2-methylenecyclopropan-1-ol, a possible product from the oxidation of methylenecyclopropane, was not available and so butan-1-ol was used as a pseudostandard. Unfortunately, this result could not be consistently reproduced. In the majority of the cases no detectable product was seen.

1-Methyl-1-phenylcyclopropane was not oxidised by the M.C. system, whereas a mixture of *cis* and *trans* 1-methyl-2-phenylcyclopropane was found to be oxidised. Although lack of time precluded the rigorous determination of the identity of the products of oxidation of 1-methyl-2-phenylcyclopropane, the t.l.c. characteristics of the product suggest that it arises *via* benzene ring hydroxylation, probably at the para position.

The conclusions that can be drawn about the topography of the enzyme's active site from the results of oxidation of these substituted cyclopropanes will be dealt with in a later Chapter.

## 2.6

### SYNTHESES OF SUBSTRATES AND POTENTIAL PRODUCTS OF ENZYMIC OXIDATION

Cyclopropanol was synthesised by the method of Depuy that is outlined in Scheme 2.7<sup>29</sup>. This involved opening of the epoxide ring of 1,2-epoxy-3-chloropropane with magnesium bromide to give an intermediate magnesium alkoxide. Ring closure to a cyclopropane was then achieved by 1,3-dehalogenation induced by finely divided iron (from treating ferric chloride with ethyl magnesium bromide).

The method used for the synthesis of methylcyclopropane, shown in Scheme 2.2<sup>30</sup>, involved addition of 9-BBN across the double bond of 2-methyl-3-chloroprop-1-ene followed by basic hydrolysis.

cyclopropane with the M.C. enzyme system and on one occasion this appeared to be successful, giving a product that had a GLC retention time similar to that of butan-1-ol. Authentic 2-methylenecyclopropan-1-ol, a possible product from the oxidation of methylenecyclopropane, was not available and so butan-1-ol was used as a pseudostandard. Unfortunately, this result could not be consistently reproduced. In the majority of the cases no detectable product was seen.

1-Methyl-1-phenylcyclopropane was not oxidised by the M.C. system, whereas a mixture of *cis* and *trans* 1-methyl-2-phenylcyclopropane was found to be oxidised. Although lack of time precluded the rigorous determination of the identity of the products of oxidation of 1-methyl-2-phenylcyclopropane, the t.l.c. characteristics of the product suggest that it arises *via* benzene ring hydroxylation, probably at the para position.

The conclusions that can be drawn about the topography of the enzyme's active site from the results of oxidation of these substituted cyclopropanes will be dealt with in a later Chapter.

## 2.6 SYNTHESES OF SUBSTRATES AND POTENTIAL PRODUCTS OF ENZYMIC OXIDATION

Cyclopropanol was synthesised by the method of Depuy that is outlined in Scheme 2.2<sup>29</sup>. This involved opening of the epoxide ring of 1,2-epoxy-3-chloropropane with magnesium bromide to give an intermediate magnesium alkoxide. Ring closure to a cyclopropane was then achieved by 1,3-dehalogenation induced by finely divided iron (from treating ferric chloride with ethyl magnesium bromide).

The method used for the synthesis of methylcyclopropane, shown in Scheme 2.2<sup>30</sup>, involved addition of 9-BBN across the double bond of 2-methyl-3-chloroprop-1-ene followed by basic hydrolysis.

But-3-en-1-ol was prepared by heating 3-cyanoprop-1-ene in acidic medium to give the but-3-enoic acid, which was reduced with  $\text{LiAlH}_4$ .

The preparation of methylenecyclopropane followed the method of Koster *et al.*<sup>31</sup> and is outlined in Scheme 2.3. This involved the treatment of 2-methyl-3-chloroprop-1-ene with sodamide to give a mixture of methylenecyclopropanes and 1-methylcyclopropene. Isomerisation of the 1-methylcyclopropene to methylenecyclopropane was achieved using potassium *tert*-butoxide in *tert*-butanol. The resulting methylenecyclopropane contained only a trace of methylcyclopropane.

The synthesis of 1-methyl-1-phenylcyclopropane<sup>32</sup> (shown in Scheme 2.4) used the phase transfer catalyst benzyltri-*N*-butylammonium bromide to produce from chloroform dichlorocarbene, which is added to the double bond of  $\alpha$ -methylstyrene to give 1-methyl-1-phenyl-2,2-dichlorocyclopropane. This dichloro intermediate was reduced by sodium in liquid ammonia to a mixture of compounds. Along with the desired product there was also 2-phenylbutane present, arising from reductive opening of the cyclopropyl ring. This ring-opened product was seen even when the amount of sodium was reduced from four to three mol equivalents. A possible mechanism, based on the Birch reduction, that could give rise to this product is shown in Scheme 2.5.

The synthesis of 1-methyl-2-phenylcyclopropane initially used the Wittig reaction of the ylid from triphenylethylphosphonium iodide with benzaldehyde to give  $\beta$ -methylstyrene (outlined in Scheme 2.6). The conversion of  $\beta$ -methylstyrene into the desired cyclopropane then followed a similar course as for 1-methyl-1-phenylcyclopropane.



## 2.7 EXPERIMENTAL

### Cyclopropanol

To a mixture of magnesium (3.6 g, 0.15 mol) and bromine (24 g, 0.15 mol) stirred in anhydrous diethyl ether (100 cm<sup>3</sup>) was added ferric chloride hexahydrate (0.15 g, 0.56 mol). 1,2-Epoxy-3-chloropropane (14 g, 0.15 mol) was then added dropwise over a period of 30 min with continual stirring. Stirring was continued for one hour and then ethyl magnesium bromide (from ethyl bromide [49 g] and magnesium [10.8 g] in anhydrous diethyl ether [400 cm<sup>3</sup>]) was added dropwise over a period of 2 h. The mixture was stirred for approximately 16 h, and then poured onto iced ammonium chloride solution (50 cm<sup>3</sup>, 16% w/v). The whole was filtered and the ether layer was extracted with water (8 x 50 cm<sup>3</sup>). The combined aqueous extracts were continually extracted with diethyl ether for ca. 96 h. The ether layer was separated, dried (MgSO<sub>4</sub>), and filtered. The majority of the ether was removed by distillation through a 42 x 2 cm Dufton column. The residue was then purified by preparative GLC (20% 20 M Carbowax on Chromosorb A, 130°C) which gave cyclopropanol as a colourless liquid. This material was homogenous by GLC.

<sup>1</sup>H NMR (CDCl<sub>3</sub> p.p.m.) 0.41 (m, 4 H), 3.35 (m, 1 H), 3.45 (broad s, 1 H).

The peak at 3.45 was removed by a D<sub>2</sub>O shake.

Major I.R. peaks (cm<sup>-1</sup>): 3610, 3300, 3090, 3010, 1030.

### Methylcyclopropane

To a stirred solution of 9-BBN (6.9 g, 56.5 x 10<sup>-3</sup> mol) in dry tetrahydrofuran (100 cm<sup>3</sup>) under a nitrogen atmosphere,

2-methyl-3-chloropropene ( $5 \text{ cm}^3$ ,  $51.3 \times 10^{-3} \text{ mol}$ ) was added dropwise over a period of 15 min. Stirring was continued for 5 h after which the intermediate organoborane was hydrolysed by addition of 3 M NaOH solution ( $20 \text{ cm}^3$ ) over a 15 min period. The product was collected overnight, after passing through a water trap, in an acetone/dry ice trap. Final purification was carried out by preparative GLC (20% DEGS on Chromosorb 80/100,  $60^\circ\text{C}$ ), which gave methylcyclopropane as a colourless liquid (stored at  $-80^\circ\text{C}$ ).

$^1\text{H}$  NMR ( $\text{CDCl}_3$  p.p.m.) 0.05 (d, 2 H), 0.50 (d, 2 H), 0.77 (m, 1 H), 1.50 (d, 3 H).

#### But-3-en-1-ol

1-Cyanobut-3-ene (3.1 g,  $46.3 \times 10^{-3} \text{ mol}$ ) was added to conc. HCl ( $9.3 \text{ cm}^3$ ) and stirred at  $60^\circ\text{C}$  for 2 h, giving a white coloured precipitate. Water ( $10 \text{ cm}^3$ ) was added, which dissolved the precipitate, and the mixture was heated at reflux temperature for 90 min. The mixture was cooled and extracted with dichloromethane ( $3 \times 10 \text{ cm}^3$ ) which was dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator. Distillation (20 mmHg,  $77-84^\circ\text{C}$ ) gave 2.7 g of but-3-en-1-oic acid.

A solution of but-3-en-1-oic acid (1.97 g,  $22.9 \times 10^{-3} \text{ mol}$ ) in diethyl ether ( $20 \text{ cm}^3$ ) was added to a stirred solution of lithium aluminium hydride (2.32 g,  $61 \times 10^{-3} \text{ mol}$ ) in diethyl ether ( $20 \text{ cm}^3$ ) at such a rate that the solution gently refluxed. The mixture was stirred overnight at room temperature. Water ( $10 \text{ cm}^3$ ) was added slowly to decompose residual reductant, followed by ice-cold  $\text{H}_2\text{SO}_4$  until the precipitate dissolved. The mixture was extracted with diethyl ether ( $3 \times 10 \text{ cm}^3$ ), dried ( $\text{MgSO}_4$ ), filtered and distilled ( $110^\circ\text{C}$ )

giving a colourless solution whose infra-red spectrum showed amine and nitrile impurities. Final purification was achieved by preparative GLC (20% DEGS, 150°C).

$^1\text{H}$  NMR ( $\text{CCl}_4$  p.p.m.) 2.23 (q, 2 H), 3.55 (t, 2 H), 4.02 (broad s, 1 H), 5.00 (m, 2 H), 5.7 (m, 1 H).

Main I.R. peaks ( $\text{cm}^{-1}$ ): 3340, 3090, 2920, 1830, 1636.

#### Methylenecyclopropane

Sodamide was prepared by adding sodium (6.9 g, 0.3 mol) over a 30 min period to sodium-distilled ammonia (ca. 400  $\text{cm}^3$ ) followed by a few crystals of ferric nitrate. The excess of ammonia was allowed to evaporate overnight and the residual sodamide was taken up in dioxan (30  $\text{cm}^3$ ). The solution was heated to reflux temperature and water (0.1  $\text{cm}^3$ ) was added, followed by 2-methyl-3-chloropropene (17.5  $\text{cm}^3$ , 0.18 mol) added dropwise over a period of 3 h. Heating and stirring was continued for a further hour. The product was carried by a slow argon stream through a series of traps containing first concentrated  $\text{H}_2\text{SO}_4$ , then NaOH pellets, followed by a solution of *t*-BuOK (2.9 g,  $25.9 \times 10^{-3}$  mol) and *t*-BuOH (2.4  $\text{cm}^3$ ) in DMSO (10  $\text{cm}^3$ ). The colourless liquid was finally collected in an acetone/dry ice trap and stored at -80°C.

$^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.) 1.02 (s, 4 H), 5.30 (s, 2 H).

#### 1-Methyl-1-phenylcyclopropane

A mixture of 1-methylstyrene (45.5 g, 0.38 mol), ethanol-free chloroform (47.4 g, 0.4 mol), sodium hydroxide solution (78  $\text{cm}^3$ , 50% w/v) and benzyl-tri-*N*-butylammoniumbromide (3.07 g,  $8.98 \times 10^{-3}$  mol)

was stirred vigorously under a nitrogen atmosphere, at reflux temperature, for 48 h. The aqueous phase was removed and extracted with dichloromethane ( $4 \times 50 \text{ cm}^3$ ) and the combined organic phases were washed with water ( $50 \text{ cm}^3$ ), dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator. Reduced pressure distillation (1.5 mmHg,  $84-85^\circ\text{C}$ ) yielded 20.03 g (25.9%) of 1-methyl-1-phenyl-2,2-dichlorocyclopropane.  $^1\text{H}$  NMR ( $\text{CCl}_4$  p.p.m.) 1.6 (s, 3 H), 1.7 (m, 2 H), 7.2 (s, 5 H).

1-Methyl-1-phenyl-2,2-dichlorocyclopropane (20 g,  $99.5 \times 10^{-3}$  mol) was taken up in dry tetrahydrofuran ( $200 \text{ cm}^3$ ) to which was added sodium-distilled ammonia (ca.  $500 \text{ cm}^3$ ). To this stirred solution sodium (9.71 g, 0.42 mol) was added over a period of 30 min. The ammonia was allowed to evaporate overnight through an iced dichloromethane trap. Residual sodium was destroyed by careful addition of water ( $20 \text{ cm}^3$ ) and the contents of both flasks were combined. The aqueous phase was removed and extracted with dichloromethane ( $4 \times 50 \text{ cm}^3$ ) and the combined dichloromethane phases were dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator. Reduced pressure distillation (15 mmHg,  $60-62^\circ\text{C}$ ) gave a mixture of 1-methyl-1-phenylcyclopropane and 2-phenylbutane. The mixture was purified by preparative GLC (20% DEGS,  $140^\circ\text{C}$ ) giving 1-methyl-1-phenylcyclopropane as a colourless liquid.

$^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.) 0.78 (m, 4 H), 1.39 (s, 3 H), 7.2 (m, 5 H).

Main I.R. peaks ( $\text{cm}^{-1}$ ) 3080, 3000, 2950, 1605, 1500, 1450, 1010, 690.

#### 1-Methyl-2-phenylcyclopropane

A mixture of triphenylethylphosphoniumiodide (166 g, 0.39 mol), dichloromethane ( $200 \text{ cm}^3$ ), sodium hydroxide solution ( $200 \text{ cm}^3$ , 50% w/v) and benzaldehyde (41.39 g, 0.39 mol) was stirred at room

temperature for 42 h. The resultant suspension was broken down by steam distillation and the aqueous layer extracted with dichloromethane ( $4 \times 50 \text{ cm}^3$ ). The combined organic layers were dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator. Unreacted benzaldehyde was removed by shaking with sodium metabisulphite solution ( $50 \text{ cm}^3$ , 30% w/v). Reduced pressure distillation (14 mmHg,  $63\text{--}65^\circ\text{C}$ ) gave 15.8 g ( $0.13 \text{ mol}$ , 34%) of a mixture of *cis*- and *trans*- $\beta$ -methylstyrene.

$^1\text{H}$  NMR ( $\text{CCl}_4$  p.p.m.) 1.75 (s, 3 H), 1.85 (s, 3 H), complex multiplet centred at 6.00 (4 H), 7.2 (s, 10 H).

1-Methylstyrene (15.3 g,  $0.13 \text{ mol}$ ), ethanol-free chloroform ( $54 \text{ cm}^3$ ), benzyl-tri-*N*-butylammoniumbromide (0.97 g,  $2.7 \times 10^{-3} \text{ mol}$ ) and sodium hydroxide solution (53.5 g, 50% w/v) were stirred at  $52^\circ\text{C}$  for 24 h. Centrifugation was used to break down the suspension and the aqueous layer was removed and extracted with dichloromethane ( $4 \times 25 \text{ cm}^3$ ). The combined organic layers were dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator. Reduced pressure distillation (13 mmHg,  $116\text{--}118^\circ\text{C}$ ) gave 25.3 g ( $0.13 \text{ mol}$ , 96.8% yield) of 1-methyl-2-phenyl-3,3-dichlorocyclopropane.

$^1\text{H}$  NMR ( $\text{CCl}_4$  p.p.m.) 1.15 (d, 3 H), 1.4 (d, 3 H), 1.9 (m, 2 H), 2.35 (d, 1 H), 2.8 (d, 1 H), 7.2 (s, 10 H).

Ratio of *trans*:*cis* (from NMR integral) 1.75:1  $J_{\text{trans}} = \text{ca. } 12 \text{ Hz}$ ,  
 $J_{\text{cis}} = \text{ca. } 8.7 \text{ Hz}$ .

To 1-methyl-2-phenyl-3,3-dichlorocyclopropane (23.9 g,  $0.12 \text{ mol}$ ) in dry tetrahydrofuran ( $250 \text{ cm}^3$ ) was added sodium-distilled ammonia (ca.  $600 \text{ cm}^3$ ). The mixture was stirred and sodium (8.99 g,  $0.39 \text{ mol}$ ) was added over a period of 30 minutes. The ammonia was allowed to evaporate overnight through an iced dichloromethane trap. The combined aqueous phases were extracted with dichloromethane ( $4 \times 30 \text{ cm}^3$ ) and the combined dichloromethane fractions were dried ( $\text{MgSO}_4$ ),

filtered and the solvent removed by rotary evaporator. Reduced pressure distillation (14 mmHg, 68-71°C) gave 3.55 g of a mixture of *cis* and *trans* 1-methyl-1-phenylcyclopropane along with 1-methyl-2-phenylpropane and 1-phenylbutane. The sample was purified by preparative GLC (Carbowax, 180°C) to give a colourless mixture of *cis* and *trans* 1-methyl-2-phenylcyclopropane (relative quantities of 1:3).

<sup>1</sup>H NMR (CDCl<sub>3</sub> p.p.m.) 0.85 (m, 6 H), 0.8 (d, 3 H), 1.15 (d, 3 H), 1.55 (m, 1 H), 2.1 (m, 1 H), 7.15 (m, 10 H).

Main I.R. peaks (cm<sup>-1</sup>): 2920, 1600, 1450, 680.

#### 2,4-Dinitrophenylhydrazone of propionaldehyde

To a solution of 2,4-dinitrophenylhydrazine in HCl (0.4%, 25 cm<sup>3</sup>) was added propionaldehyde (0.5 cm<sup>3</sup>). The mixture was stirred at room temperature for 30 minutes, filtered and the crude product was recrystallised from methanol to give yellow crystals, m.p. 155°C (lit. m.p. 155°C)<sup>33</sup>. T.l.c. analysis (toluene/pet. ether [40-60 fraction], silica gel, u.v.) showed a single spot of R<sub>f</sub> 0.13.

#### 2,4-Dinitrophenylhydrazone of acetone

This was prepared in the manner described for propionaldehyde. to give yellow crystals m.p. 128°C (lit. m.p. 128°C)<sup>33</sup>. T.l.c. analysis (toluene/pet. ether [40-60 fraction], silica gel, u.v.) showed a single spot of R<sub>f</sub> 0.09.

#### α-Naphthylurethane of cyclopropylcarbinol

α-Naphthylisocyanate (1.69 g, 10 × 10<sup>-3</sup> mol) and cyclo-

propylcarbinol ( $72 \times 10^{-3}$  g,  $1 \times 10^{-3}$  mol) were mixed and heated on a water bath for 10 min, cooled, and the resultant solid broken up with a glass rod. Recrystallisation from petrol-ether (100-120 fraction) gave white coloured crystals with a melting point of  $116^{\circ}\text{C}$  (lit. M.P.  $119.5$ )<sup>34</sup>. T.l.c. analysis ( $\text{CH}_2\text{Cl}_2$ , silica gel, u.v.) showed a single spot of  $R_f$  0.28.

$^1\text{H}$  NMR ( $\text{CCl}_4$  p.p.m.) 0.35 (m, 2 H), 0.6 (m, 2 H), 1.2 (m, 1 H), 6.05 (d, 2 H), 7.0 (broad s, 1 H), 7.7 (m, 7 H).

#### Benzyltri-N-butylammonium bromide

Tri-N-butylamine (12.9 g,  $69.6 \times 10^{-3}$  mol) and benzylbromide (11.8 g,  $69.6 \times 10^{-3}$  mol) were mixed in diethyl ether ( $10 \text{ cm}^3$ ) and stirred at room temperature overnight. The product precipitated as a white powder that was collected by filtration, washed with diethyl ether and air-dried giving 21.9 g ( $61.5 \times 10^{-3}$  mol, 88% yield).

#### Triphenylethylphosphonium iodide

Triphenylphosphine (154.7 g, 0.59 mol) was dissolved in benzene ( $400 \text{ cm}^3$ ) and ethyl iodide (91.5 g, 0.59 mol) was added, the mixture was stirred at  $45^{\circ}\text{C}$  for 48 h. The white precipitate was collected by filtration and air-dried giving 157.6 g (0.38 mol, 63.9% yield).

#### Derivatisation of the Enzymic Oxidation Product of Cyclopropane

After the oxidation of cyclopropane (described in Materials and Methods) the resultant mixture was divided into two equal portions and

to both was added an acidic DNP solution (0.4%, 25 cm<sup>3</sup>). While one sample was stirred at room temperature for 45 min, the second one was stirred at 70°C for 45 min. They were then worked up individually by extraction with dichloromethane (3 x 25 cm<sup>3</sup>) which was dried (MgSO<sub>4</sub>), filtered and the solvent removed by rotary evaporator. The red coloured residue was triturated in diethylether (2 cm<sup>3</sup>) and then pet. ether (40-60 fraction, 2 cm<sup>3</sup>) was added. The mixture was filtered through a short silica gel column using diethyl ether: pet. ether (40-60 fraction) 1:1 as eluant. The solvent was removed from the eluate by rotary evaporator.

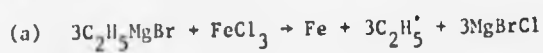
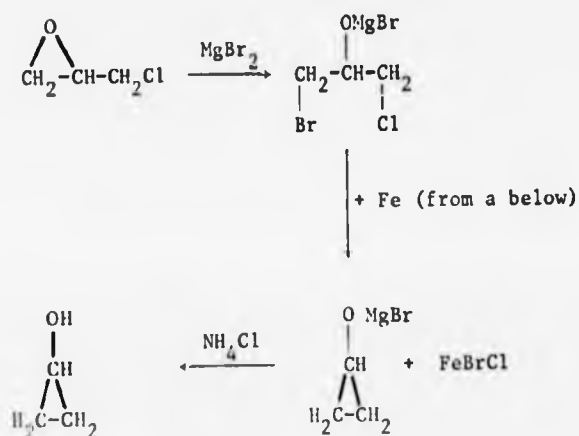
#### Derivatisation of the Enzymic Oxidation Product of Methylcyclopropane

The product was isolated by continuous extraction using diethyl ether (ca. 100 cm<sup>3</sup>) for a period of 48 h. The ether was then removed, dried (MgSO<sub>4</sub>), filtered and the volume reduced to ca. 2 cm<sup>3</sup> by distillation. This volume was further reduced to ca. 200 µl by carefully blowing off the ether with nitrogen. The product was then taken up in pet. ether (50-60 fraction, 3 cm<sup>3</sup>) and α-naphthylisocyanate (20 µl) was added. The mixture was then heated at reflux temperature overnight. The unreacted α-naphthylisocyanate was decomposed by the addition of water (10 cm<sup>3</sup>) and stirring for 4 h. The mixture was then heated to its boiling point and the pet. ether fraction was removed, the aqueous phase was then extracted twice more with hot pet. ether (5 cm<sup>3</sup>). The combined pet. ether fractions were dried (MgSO<sub>4</sub>), filtered and the solvent removed by rotary evaporator. The product was dissolved in dichloromethane and purified by preparative t.l.c. (CH<sub>2</sub>Cl<sub>2</sub>, silica gel, u.v.).

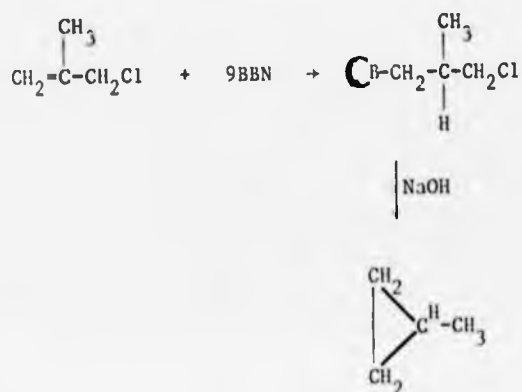
<sup>1</sup>H NMR discussed in Section 4.



## SCHEME 2.1

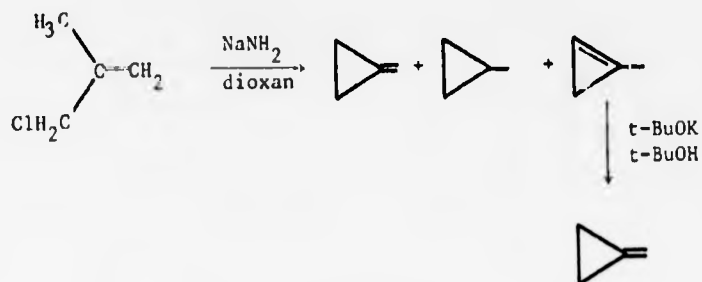


## SCHEME 2.2



9BBN = 9-Bora-bicyclo[3.3.1]nonane

## SCHEME 2.3

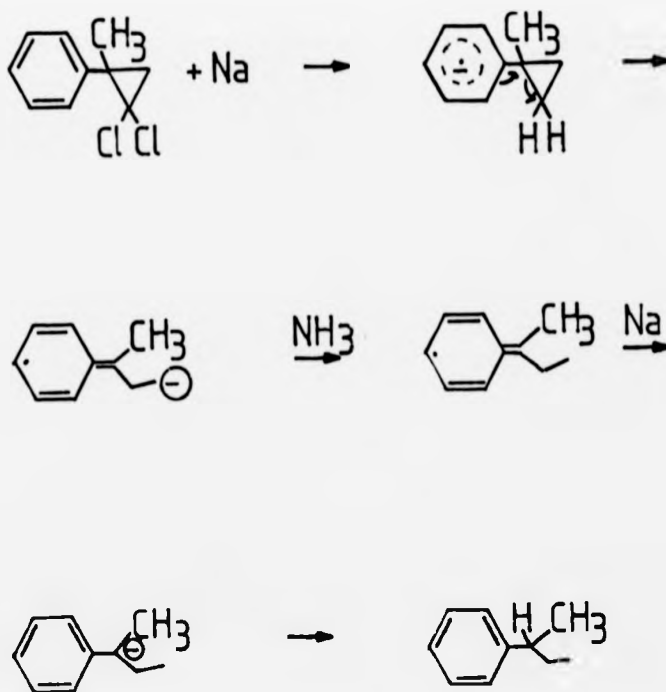


## SCHEME 2.4

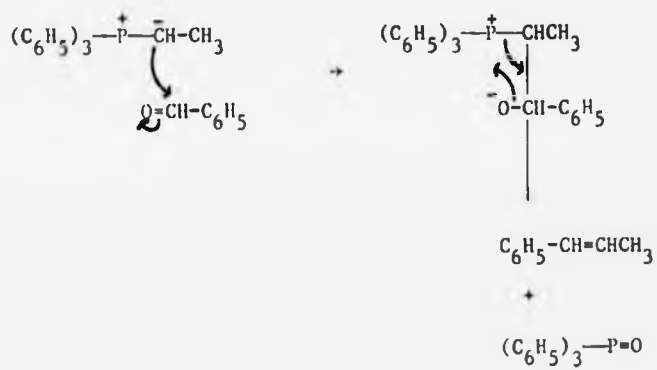
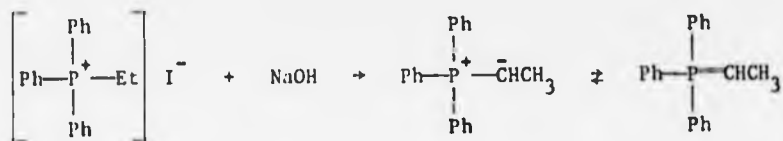


SCHEME 2.5

Possible mechanism for the ring opening reaction found in the synthesis of the methyl, phenyl substituted cyclopropanes.



## SCHEME 2.6



## CHAPTER 2 - REFERENCES

1. 'Advanced Organic Chemistry', II Ed., J. March, 1977 McGraw-Hill, and references contained therein
2. 'Organic Chemistry', Morrison and Boyd, 1974, Allyn and Bacon, and references contained therein
3. 'Comprehensive Organic Chemistry', Vol. I, T. Clark, 1979, Pergamon Press, and references contained therein
4. H. Kabuichi, *Bull. Chem. Soc. Japan*, 1973, 46, 1568
5. A. A. Scala and W. T. Wu, *J. Phys. Chem.*, 1970, 74, 1852
- 5.(a) D. J. Bogan and C. W. Hand, *J. Phys. Chem.*, 1978, 82, 2067
6. W. K. Stuckey and J. Heicklen, *J. Chem. Phys.*, 1967, 46, 4843
- 6.(a) R. E. Huie and J. T. Herron, *Prog. React. Kinet.*, 1975, 8, 1
7. C. C. Lee and L. Gruber, *J. Am. Chem. Soc.*, 1968, 90, 3775
8. C. C. Lee, *et al.*, *J. Org. Chem.*, 1969, 34, 3210
9. 'Carbonium Ions', Vol. III, Ed. G. A. Olah and P. von R. Schleyer, 1972, Wiley Interscience: Ch. 25 - Herman P. Rickey Jr.; Ch. 26 - K. B. Wiberg, B. Andes Hess Jr., A. J. Ashe III, and references contained therein
10. G. A. Olah, R. J. Spear, P. C. Hiberty and W. J. Hehre, *J. Am. Chem. Soc.*, 1976, 98, 7470
11. M. J. S. Dewar and S. Kirshner, *ibid*, 1974, 96, 5244
12. 'Free Radicals', Vol. I, J. K. Kochi, 1973, J. Wiley and Sons and references contained therein
13. R. W. Fessenden and R. H. Schuler, *J. Chem. Phys.*, 1963, 39, 2147
14. K. Kabayashi and J. B. Lambert, *J. Org. Chem.*, 1977, 42, 1254
15. H. M. Walborsky, *Tetrahedron*, 1981, 37, 1625
16. B. Maitland, D. Forrest and K. H. Ingold, *J. Am. Chem. Soc.*, 1976, 98, 7024
17. J. K. Kochi, P. J. Krussie and D. R. Eaton, *ibid*, 1969, 91, 1877
18. R. A. Sheldon and J. K. Kochi, *ibid*, 1970, 92, 4395
19. E. Renk, *et al.*, *ibid*, 1961, 83, 1987
20. H. Hart and R. A. Cipriani, *ibid*, 1962, 84, 3697

21. 'Fundamentals of Carbanion Chemistry', D. J. Cram, 1965, Academic Press, and references contained therein
22. Rodds 'Chemistry of Carbon Compounds', 2nd Ed., Pt. A, Ed. S. Coffey, Elsevier Publishing Co., and references contained therein
23. W. H. Walborsky, F. J. Imparato and A. E. Young, *J. Am. Chem. Soc.*, 1964, 86, 3283
24. D. E. Applequist and A. H. Peterson, *ibid*, 1961, 83, 862
25. J. Ooyama and J. W. Foster, *Antonie van Leeuwenhoek*, 1965 31, 45
26. C. H. Depuy, *Acc. Chem. Res.*, 1968, 1, 33
27. J. van Eyk and T. J. Bartels, *J. Bacteriol*, 1968, 706
28. H. Dalton, *et al.*, *J. Chem. Soc. Chem. Comm.*, 1981, 482
29. C. H. Depuy, G. H. Dappen, K. L. Eiders and R. A. Klein, *J. Org. Chem.*, 1964, 29, 2813
30. 'Organic Synthesis via Boranes', H. C. Brown, 1975, Wiley Interscience, and references contained therein
31. R. Koster, S. Arona and P. Binger, *Synthesis*, 1971, 322
32. T. Hiyama, H. Sawada, M. Tsuhanaha and H. Nogaki, *Tetrahedron Lett.*, 1975, 34, 3013
33. 'CRC Handbook of Tables for Organic Compound Identification', 3rd Edition, CRC Press
34. S. Sarel and M. S. Newan, *J. Am. Chem. Soc.*, 1956, 78, 5416

## CHAPTER 3

### ARENES

#### 3.1 OBJECTIVES

The major objective of the work described in this Chapter was to determine if an 'NIH shift' accompanied the hydroxylation of aromatic compounds by the M.C. monooxygenase.

A secondary objective was to determine how a change in the substituent group on the arene affected both the regiospecificity and efficiency of this oxidation.

#### 3.2 NON-ENZYMIC AROMATIC AND ALIPHATIC SUBSTITUTION

Aromatic substitution, whether electrophilic or nucleophilic, normally involves a stepwise reaction mechanism of addition to give an intermediate  $\sigma$ -complex followed by elimination<sup>1</sup>. Other rarer mechanisms are also known such as that involving a benzyne intermediate. Free radical substitutions of alkyl-substituted aromatic compounds normally take place on the carbon  $\alpha$  to the aromatic ring (*cf.* photochemical halogenation), because in this position maximum stabilisation of the transition state can be achieved. Many free radical substitutions of aromatic rings are known. Examples of these processes are the aminations by  $R_2NCl$ <sup>2</sup>, which can be effected thermally or photochemically, or by catalysis with  $AlCl_3$  or a redox metal such as  $Fe^{II}$ ,  $Ti^{III}$ ,  $Cu^I$ , or  $Cr^{II}$ . A well established example of aromatic hydroxylation *via* a radical mechanism is seen in the substitution reaction of aromatic hydrocarbons with Fenton's reagent (see Chapter 1 on Model Systems).

This mechanism also involves a  $\sigma$ -complex, a hydroxycyclohexadienyl radical derived by addition of a hydroxy radical to a benzene ring. Dimerisation then results unless an oxidant such as  $\text{Cu}^{\text{II}}$ ,  $\text{Fe}^{\text{III}}$  or oxygen is present to oxidise this intermediate to a phenol. The reversibility of addition of the hydroxyl radical to the benzene ring has been demonstrated by Eberhardt<sup>3</sup>.

Similar reagents to those described above have been utilised to substitute unactivated aliphatic hydrocarbons. In a reaction with  $\text{R}_2\text{NCl}$ <sup>4</sup> the product is a chlorine-substituted hydrocarbon, in contrast to the amination product mentioned above. This process is believed to operate *via* a radical mechanism, to give, with hydrocarbons  $\leq \text{C}_6$ , a product of mono-substitution.

Non-radical substitution of aliphatic hydrocarbons has been achieved by the action of 30% aqueous  $\text{H}_2\text{O}_2$  in  $\text{TFA}$ <sup>5</sup> and also by Olah's<sup>6,7</sup> superacids. For the latter, protonation of water or oxygen with either 'magic acid' ( $\text{HSO}_3\text{F-SbF}_3$ ) or fluoroantimonic acid ( $\text{HF-SbF}_5$ ) gives electrophilic species capable of hydroxylating methane. With other hydrocarbons cleavage of their carbon chain also occurs.

It may be hoped that elucidation of the mechanisms by which enzymes hydroxylate unactivated hydrocarbons will lead to improved methods for achieving transformations of such molecules. Alternatively, clues about the enzymic mechanisms might be gleaned from observations on appropriate chemical systems.

### 3.3 THE NIH SHIFT

Analysis of the tritium lost from [4-<sup>3</sup>H]acetanilide during its oxidation by the aryl hydroxylase of liver microsomes appeared to Guroff and his co-workers<sup>8</sup> to be a relatively convenient method for



assaying this enzyme. Unfortunately, analysis of the product (p-hydroxyacetanilide) showed that tritium loss was negligible even though oxidation had obviously taken place. Some time later, attempts by these workers to use a similar method of assay for both tryptophan-5-hydroxylase and phenylalanine hydroxylase again showed unexpected retention of tritium. Faced with these results, the first reaction of Guroff *et al.* was to question the specificities of the procedures used to label the substrates. As these assays used relatively low levels of tritium labelling attempts to verify the position of incorporation would not be a trivial task. In order to overcome this problem [4-<sup>2</sup>H]phenylalanine was synthesised and <sup>1</sup>H NMR spectroscopy was used to demonstrate that deuterium occupied solely the 4-position. Oxidation of this substrate by phenylalanine hydroxylase led to tyrosine in which between 60 and 70% of the deuterium label had been retained. The conclusion drawn was that during the oxidation procedure a rearrangement had taken place causing deuterium to migrate from the 4-position to some other position<sup>8</sup>. To identify this position the deuterated tyrosine was treated with tyrosine hydroxylase to give 3,4-dihydroxyphenylalanine. This product retained 50% of the deuterium originally present in the tyrosine. Hence, the label had moved from the 4- to the 3-position during the oxidation with phenylalanine hydroxylase. This conclusion was confirmed by treating the deuterated tyrosine with N-iodosuccinamide giving 3,5-diiodotyrosine with complete loss of deuterium.

The mechanism by which the migration of deuterium or tritium occurs in the oxidation of phenylalanine by phenylalanine hydroxylase is believed to be that shown in Fig. 3.1. This involves an initial attack by some enzymic oxidising species on one of the formal double bonds of the aromatic ring to give an arene oxide ( $\text{I}$ ). This epoxide

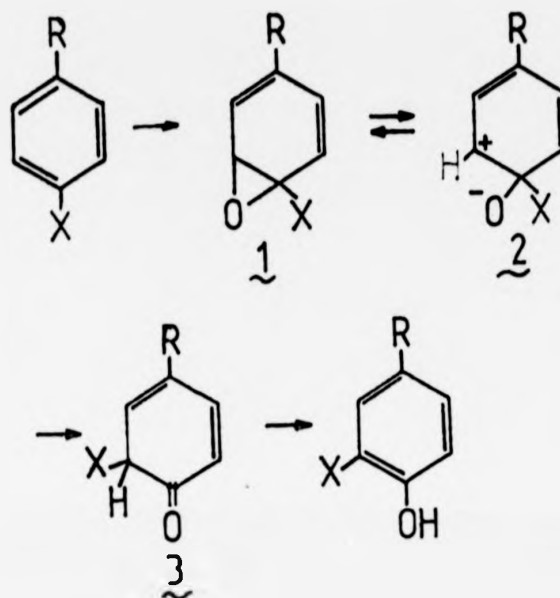


Fig. 3.1

is believed to be in equilibrium with the ring-opened zwitterion (2) from which the formation of dienone (3) is envisaged to take place by 1,2-deuterium migration. Enolisation of this dienone with loss of hydrogen or deuterium, should, if this step is non-enzymic, be dependent on a primary kinetic isotope effect. In publishing this work, Guroff and his co-workers christened the migratory mechanism the 'NIH shift', NIH standing for the National Institute of Health where the reaction was discovered.

Since its 'unveiling ceremony' the NIH shift<sup>9</sup> has been shown to occur in oxidations that are mediated by a number of mono-oxygenase enzymes from a wide variety of biological milieu including mammalian, plant, fungal and bacterial systems. Model systems have also been used to effect oxidations in which an NIH shift is seen

(see the section on Model Systems in Chapter 1).

The NIH shift has been shown to operate, for certain enzymes, with chlorine, bromine or simple alkyl moieties as the migrating group instead of hydrogen or its isotopes.<sup>9</sup>

The postulated intermediacy of an epoxide was given good experimental support when, in 1968, naphthalene was oxidised by liver microsomes<sup>10,11</sup>. The product 1-naphthol was completely separated by chromatography from a band containing unreacted naphthalene. The contents of this band were then treated with acid and gave a product spot on t.l.c. that co-chromatographed with 1-naphthol. In the chromatographic systems used naphthalene-1,2-oxide was not separated from naphthalene and so the acidic treatment of the band containing naphthalene converts any oxide present to 1-naphthol. In addition to supporting the intermediacy of an epoxide in the hydroxylation of naphthalene these results also indicated that the epoxide is an enzymic product and suggested that its rearrangement to 1-naphthol need not be enzyme-mediated. The spontaneous isomerisation of synthetic naphthalene-1,2-oxide in various solvents and buffer systems to give comparable products to those found in enzymic oxidation of naphthalene also supported this premise<sup>12</sup>.

The major product from the spontaneous isomerisation of naphthalene-1,2-oxide is 1-naphthol<sup>9,11,13</sup>. The failure to observe much 2-naphthol is believed to be due to the greater stability of the zwitterion (4, Fig. 3.2) which leads to 1-naphthol, compared to the zwitterion (5) which is a precursor of 2-naphthol<sup>12</sup>.

Indirect evidence for the presence of an intermediate ketone also came out of the work on naphthalene. When either [1-<sup>2</sup>H]- or [2-<sup>2</sup>H]naphthalene was oxidised the deuterium retention was found to be independent of the position of the label in the substrate<sup>10</sup>.

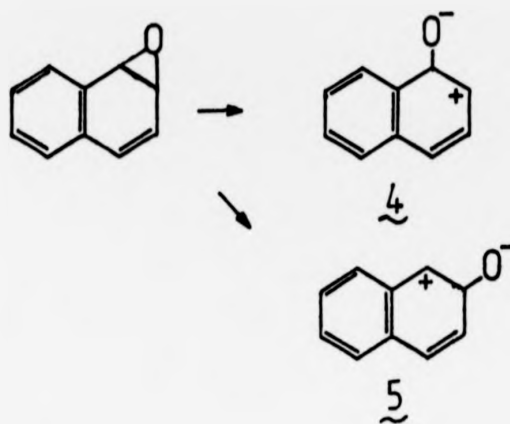


Fig. 3.2

This indicates the presence in the reaction pathway of a common intermediate, probably the ketone shown below. This ketone (undeuterated) has been observed in the photochemical rearrangement of naphthalene-1,2-oxide at  $-180^{\circ}\text{C}$ .<sup>9</sup> Also, rearrangement of 1,2-dimethylnaphthalene-1,2-oxide gives the stable 1-oxo-2,2-dimethylnaphthalene<sup>9</sup>.

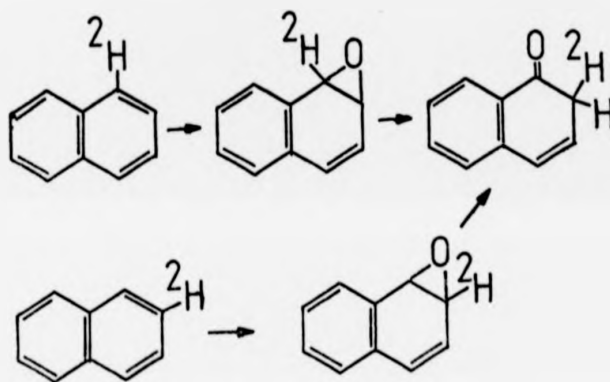


Fig. 3.3

Labelling work has shown that the enolisation of the keto intermediate is frequently a non-enzymic process, because it is normally associated with a marked primary isotope effect. If the enolisation were enzyme-induced it is expected that the proton abstraction step occurs stereospecifically (chiral enzyme acting on diastereotopic methylene hydrogens<sup>14</sup>). Thus, if one of the hydrogens of this methylene group is replaced by deuterium or tritium, depending on the stereospecificity of the process, the isotope of hydrogen would be either completely lost or retained. Tritium retentions are normally higher than deuterium retentions as is demonstrated in the oxidation of [4-<sup>3</sup>H]phenylalanine by phenylalanine hydroxylase. This gives 94% retention, whereas oxidation of [4-<sup>2</sup>H]phenylalanine gives only 70% retention<sup>15</sup>.

If the mechanism shown in Fig. 3.1 is correct and the enolisation step is a non-enzymic process, then the same level of retention should be attained irrespective of whether tritium is in the 3- or 4-position of the starting material. This assumption appears to be validated by the observations that oxidation of [4-<sup>3</sup>H]phenylalanine gives 94% tritium retention, whereas oxidation of [3-<sup>3</sup>H]phenylalanine gives 95-96% tritium retention. The importance of the primary kinetic isotope effect for the enolisation step is further demonstrated by the observations that the amount of tritium retention is changed by altering the nature of the substituent on the migratory terminus. Table 3.1 shows that if tritium moves to a carbon bearing a hydrogen then the retention is higher than if there was already a deuterium there.

If the loss of hydrogen from the keto intermediate does not take place in solution away from the enzyme but is effected by a basic group on the enzyme, then the oxidation of [1-<sup>2</sup>H]naphthalene

and [2-<sup>2</sup>H]naphthalene should in one case give complete retention and in the other case complete loss of deuterium, assuming that the epoxidation step is stereospecific (i.e. giving a pure enantiomeric naphthalene-1,2-oxide). With microsomal oxidation both of these substrates gave *ca.* 65% retention of deuterium<sup>10</sup>.

Although the vast majority of results from research on the 'NIH shift' support the postulated pathway (Fig. 3.1), there are some anomalous findings that do not completely fit into this pattern. One such result was found in the hepatic microsomal oxidation of [4-<sup>2</sup>H]chlorobenzene, where a deuterium retention of *ca.* 54% was found, whereas [3-<sup>2</sup>H]chlorobenzene proceeds with *ca.* 93% retention<sup>15</sup>. This could mean that either some deuterium is lost before the rearrangement to ketone (*cf.* Fig. 3.4) or that there is an enzyme-mediated removal preferentially, though not exclusively, of one of the atoms from a chiral intermediate (*cf.* Fig. 3.5).

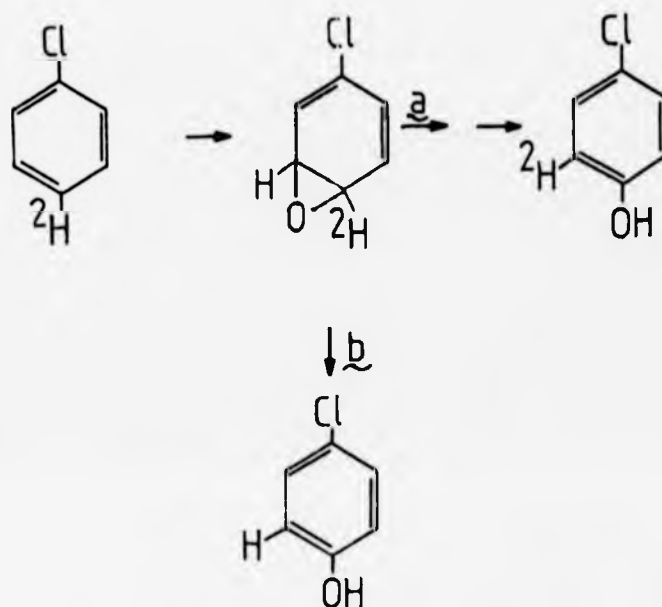
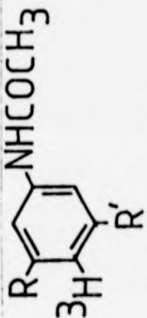
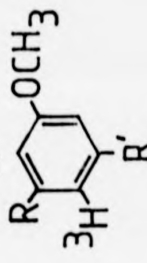
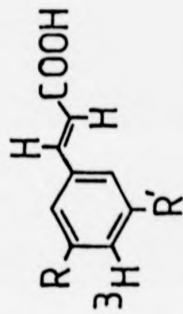


Fig. 3.4

TABLE 3.1 The Effect of Adjacent Deuteriums on the Retention of Tritium in 4-Hydroxylated Products Derived from 4-Tritiated Aromatic Substrates

Substrate	Product	System	Percent Tritium in Product		
			I R', R = H	II R, R' = $^2\text{H}$	I/II
	4-OH	Rat, <i>in vivo</i> Rat microsomes	40 62	26 40	1.54 1.55
	4-OH	Rat, <i>in vivo</i> Rat microsomes Guinea pig microsomes	77 80 79	61 64 63	1.26 1.25 1.26
	4-OH	Chick Pea microsomes	90-92	67-68	1.33

Reproduced from D. J. Reed, *et al.*, *Arch. Biochem. Biophys.*, 1973, 154, 642

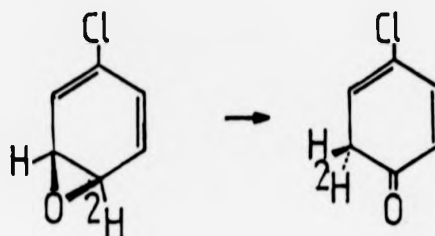


Fig. 3.5

The fact that this oxidising system does not normally show results consistent with the latter possibility must argue for a loss of deuterium before the ketone is formed. This is supported by a number of oxidations with the same enzyme system on different substrates. The pattern that emerges is that the nature of the R group on the substrate influences the amount of deuterium retained<sup>9</sup>. Ionisable or electron donating substituents capable of stabilising the intermediate cationic species enable direct loss of deuterium *via* pathway b of Fig. 3.4 to compete more effectively and so result in generally lower values of deuterium retention. As may be expected the deuterium or tritium retention found when the R substituent is an ionisable group (e.g.  $\text{NH}_3^+$ ) is a function of the pH of the medium; the more basic the medium the lower the deuterium retention<sup>9</sup>.

The final supportive evidence that the 'NIH shift' is not totally enzyme-mediated comes from the observation that the amount of tritium retained in the oxidation of [4-<sup>3</sup>H]phenylalanine by phenylalanine hydroxylase is not species-specific. The amount of retention observed for the monooxygenase enzymes derived from *Pseudomonas*, *Penicillium* or liver microsomes is > 90%<sup>9</sup>.



## 3.4

ENZYMIC OXIDATION OF ARENES

In an attempt to find a substrate for the monooxygenase from *Methylococcus capsulatus* with the structure shown in Fig. 3.6 (the reason for which will be explained in Chapter 4) it was decided to try  $\alpha$ -methylstyrene. This substrate was chosen on account of its convenient molecular size and hence handling characteristics of its likely product(s) of oxidation.

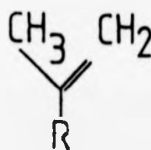
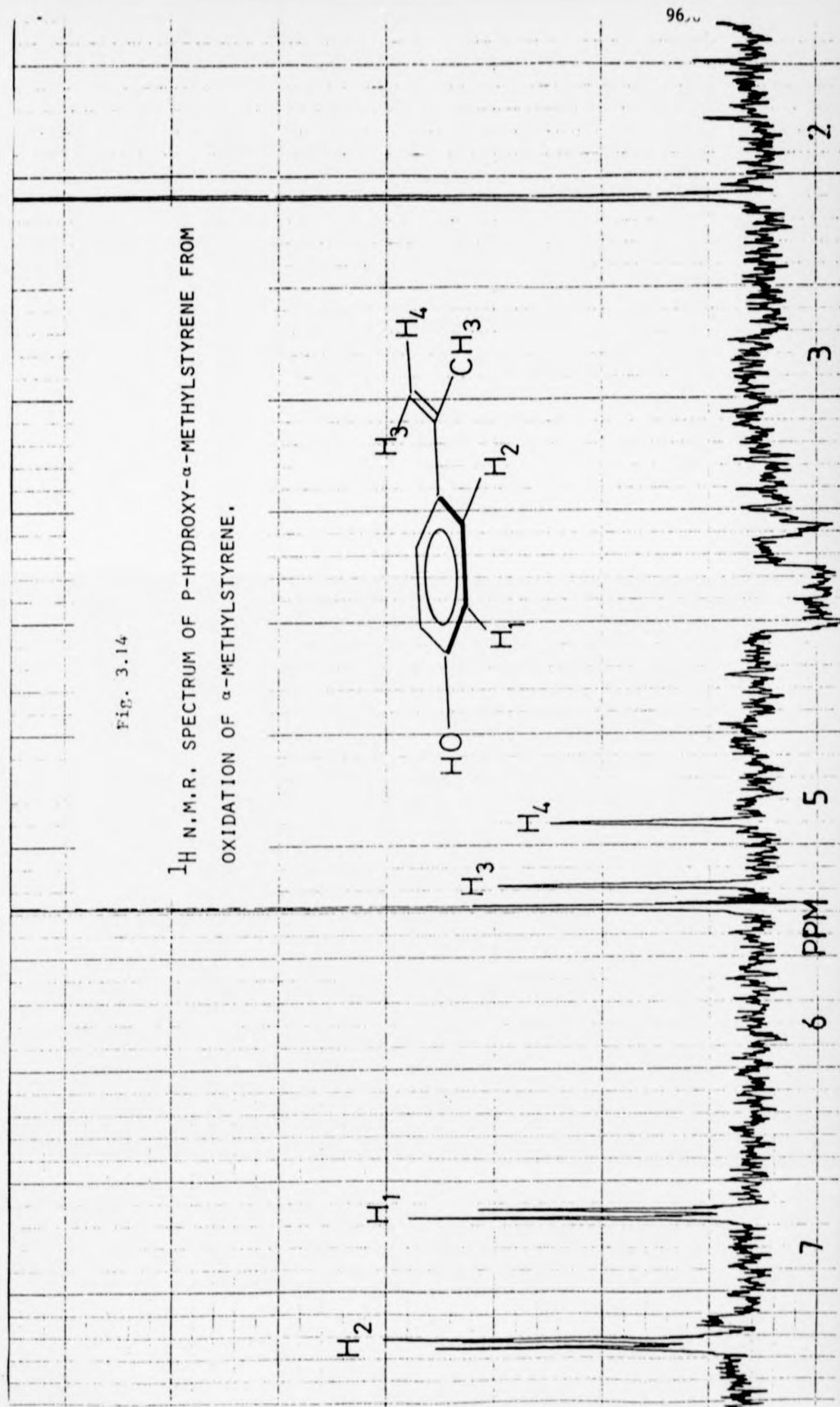


Fig. 3.6

Oxidation of  $\alpha$ -methylstyrene with the monooxygenase from *M. capsulatus* gave a product which could not be identified by A. K. Wong<sup>16</sup>, although a number of likely products of oxidation (2-methyl-2-phenyloxiran, 2-phenylprop-2-en-1-ol and 2-phenylprop-2-en-1-al) had been synthesised to provide authentic standards for t.l.c. The author re-examined this problem by carrying out a small scale oxidation that showed a single product spot on t.l.c. analysis. Increasing the scale of the oxidation and purifying this product by preparative t.l.c. enabled a <sup>1</sup>H NMR spectrum to be obtained. This spectrum, shown in Fig. 3.14, shows a singlet methyl peak at 2.1 p.p.m. and for the olefinic hydrogens, two singlets at 4.9 and 5.2 p.p.m. The large peak at 5.3 p.p.m. is due to contamination with dichloromethane. The A<sub>2</sub>B<sub>2</sub> quartet typical of para-disubstituted aromatic compounds shows as two doublets centred on 6.65 and 7.2 p.p.m. This interpretation of the spectrum showed that the product was the phenol produced by

Fig. 3.14

$^1\text{H}$  N.M.R. SPECTRUM OF P-HYDROXY- $\alpha$ -METHYLSTYRENE FROM  
OXIDATION OF  $\alpha$ -METHYLSTYRENE.



para-hydroxylation of the aromatic ring. This conclusion was supported by the u.v. spectrum which showed a  $\lambda_{\text{max}}$  of 258 nm. Addition of one drop of 5 M NaOH resulted in a movement of this peak to 282 nm and an increase in its extinction coefficient. Addition of 1 M HCl caused the peak to move back to its original position of 258 nm with a corresponding reduction in peak height. This type of behaviour is typical of phenols, treatment with a base causing abstraction of the phenolic hydrogen (Fig. 3.7). The resultant anion is stabilised by conjugation with the aromatic ring giving a larger chromophore with concomitant increase in its  $\lambda_{\text{max}}$ . Acidification re-protonates this anion with regeneration of the phenol.

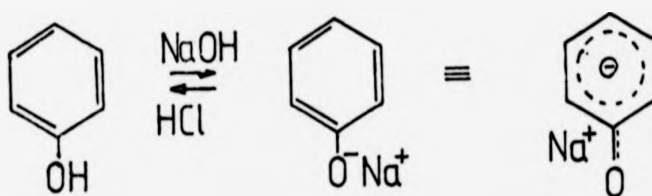


Fig. 3.7

Attempts at further characterisation of the product of enzymic oxidation of  $\alpha$ -methylstyrene by electron impact mass spectrometry were not entirely successful due, probably, to self-protonation of the phenol to give an apparent molecular ion at  $m/z$  of 145 instead of 144.

The fact that the major isolable product was the para-disubstituted arene suggested that the oxidising agent used by *M. capsulatus* is electrophilic in nature, like the species believed to participate in cytochrome P-450 oxidations.

To see if this result was repeated with other aromatic compounds we initially chose ethylbenzene as a substrate. The reason for this choice was that all possible products from mono-hydroxylation of the aromatic ring, i.e., 2-, 3- and 4-ethylphenol were readily available, as were the potential products from oxidation of the side chain, i.e., 1- and 2-phenylethanol. A small-scale oxidation was carried out and the products were analysed by t.l.c. This showed two spots which were confirmed to be products of mono-oxygenase activity by appropriate control experiments (see Materials and Methods section). One of these spots co-chromatographed with 4-ethylphenol and the other with 1-phenylethanol. Increasing the scale and analysing the products by  $^1\text{H}$  NMR spectroscopy supported these deductions. One product showed the  $A_2B_2$  coupling pattern in the aromatic region expected from 4-ethylphenol. The second product showed a singlet at  $\delta$  7.25 p.p.m. and a quartet at 4.85 p.p.m. These signals correspond to the resonances in the  $^1\text{H}$  NMR spectrum of authentic 1-phenylethanol, from its aromatic and side-chain methine, respectively. The resonance from the methyl group was masked by impurities, but the coupling constant of the methine hydrogen to this methyl group was observed to be approximately 6 Hz. A u.v. spectrum of the 4-ethylphenol showed a  $\lambda_{\text{max}}$  at 280 nm that on treatment with 5 M NaOH moved to 288 nm and returned to 280 nm on treatment with 1 M HCl. As a final method of characterisation g.l.c. analysis was used. With a column of SP1000 on Carbowax P it was possible to separate the three ethylphenol isomers. From the product of enzymic oxidation of ethylbenzene, two predominant peaks were observed corresponding to 4-ethylphenol and 1-phenylethanol. No significant peak could be seen that would be consistent with the presence of 2-phenylethanol. Also, no peaks were seen to co-chromatograph with authentic 2- and 3-ethylphenols. The accuracy of the analyses for these phenols was relatively poor because their retention times were long and peaks were rather broad. However, it

was possible to deduce that  $\leq 2\%$  2- and  $\leq 1\%$  3-ethylphenol had been formed (% of the 4-ethylphenol isomer formed).

The observed para-hydroxylations imply an electrophilic character for the enzymic oxidising agent. The low yield or absence of meta-hydroxylated product is not surprising. However, the non-formation of ortho-hydroxylated product needs some explanation. This is probably a consequence of the way the active site of the M.C. mono-oxygenase binds the substrate. If the active site is a narrow depression or cleft in the enzyme's surface and the aromatic substrate binds in one of the configurations shown in Fig. 3.8, then the sites of oxidation would be determined primarily by their relative proximity to the oxidising agent.

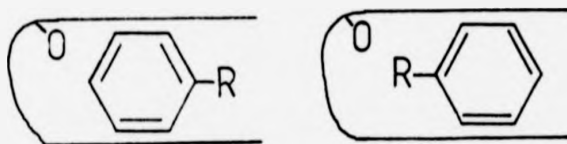


Fig. 3.8

If the binding conformation were the only criteria then one would expect, from the model shown, that ethylbenzene would give 2-phenylethanol in preference to 1-phenylethanol. That this does not occur may indicate the need for the substrate to be able to stabilise some transitory charge in the oxidation process. This point will be discussed later in this Chapter and also in Chapter 5.

Oxidation of toluene gave two product spots on t.l.c. analysis that co-chromatographed with authentic para-cresol and benzyl alcohol. Attempts to separate the 2-, 3- and 4-cresol isomers or their 3,5-dinitrobenzoates by t.l.c. was unsuccessful. Increasing the scale of the oxidation and purification of the products by

preparative t.l.c., followed by analysis by  $^1\text{H}$  NMR spectroscopy, showed the band with the higher  $R_f$  to be para-cresol (comparison of the  $^1\text{H}$  NMR spectrum with that obtained from an authentic sample). Similarly, the other product was identified as benzyl alcohol. G.l.c. analysis confirmed these findings and showed the presence of  $\leq 3\%$  2- and  $\leq 5\%$  3-cresol (% of the para-cresol formed). The ratio of benzyl alcohol to para-cresol was *ca.* 4:1 showing a marked change from the 1:1 ratio of 1-phenylethanol to 4-ethylphenol observed in the oxidation of ethylbenzene. This result is consistent with the proposed model of the active site, because increasing the length of the side-chain on the aromatic ring could result in increased steric hindrance to binding in the mode leading to side-chain oxidation (*cf.* Fig. 3.9).

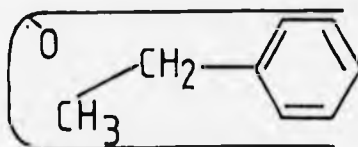


Fig. 3.9

Oxidation of styrene also led to two products. These were identified as 4-hydroxystyrene and styrene epoxide by comparison of their  $^1\text{H}$  NMR spectra with those obtained from authentic samples. The u.v. spectrum of the phenolic product showed a  $\lambda_{\text{max}}$  of 258 nm that on addition of aqueous NaOH moved to 282 nm. On acidification a peak was again observed at 258 nm. The ratio of the 4-hydroxystyrene to styrene epoxide was *ca.* 1:1 (*cf.* ethylbenzene oxidation). Oxidation of propylbenzene and butylbenzene each showed a single product spot on t.l.c. For propylbenzene, isolation of the product of oxidation by preparative t.l.c. enabled  $^1\text{H}$  NMR spectroscopic analysis to be carried out. This showed an  $A_2B_2$  pattern in the aromatic region

indicating the product to be 4-propylphenol. The oxidation of butylbenzene gave insufficient product for analysis by  $^1\text{H}$  NMR spectroscopy, but its behaviour on t.l.c. was consistent with hydroxylation having occurred at the para-position. These results are consistent with the proposed model of the active site of the M.C. mono-oxygenase (see above). The decrease in product(s) from oxidation of the alkyl residue, relative to the oxidation in the ring, found with ethylbenzene as compared to toluene, is maintained as the chain-length is increased. Indeed, for chain-lengths above  $\text{C}_2$  no definite oxidation of the side-chain could be observed. The increase in side-chain length also seems to decrease the overall efficiency of oxidation. For t-butylbenzene no oxidation products of any kind were observed. For oxidation of butylbenzene analysis of the product by  $^1\text{H}$  NMR spectroscopy in the manner used for ethylbenzene was not possible. Thus, insufficient product was formed from butylbenzene to give adequate signal-to-noise ratio in the  $^1\text{H}$  NMR spectrum. These results indicate that the active site is a crevice in a protein (*cf.* Fig. 3.10) and as the side-chain becomes either longer and more flexible, or just bulky, it prevents the aromatic portion of the molecule from reaching the oxidant efficiently. The lack of side-chain oxidation with  $\alpha$ -methylstyrene may be due to the methyl group preventing the substrate from approaching close enough to the oxidant (*cf.* Fig. 3.11).

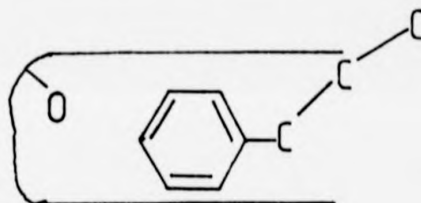


Fig. 3.10

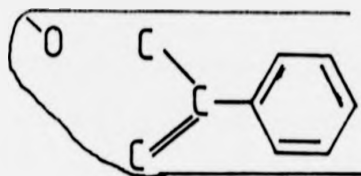


Fig. 3.11

Another potential substrate with an 'α-methyl' group is cumene and predictably oxidation of this substrate gave a single product spot on t.l.c.  $^1\text{H}$  NMR spectroscopic analysis of this product showed an  $A_2B_2$  pattern in the aromatic region again indicating para-hydroxylation. The lack of other aromatic resonances indicated that any products arising from oxidation in the propyl group were  $\leq 10\%$  of the 4-hydroxycumene product.

If, as suspected, the lower oxidation rates for substrates with a  $C_3$ , or longer, alkyl side-chain are due to steric inhibition attributable to the size and flexibility of this side-chain, then introduction of a  $C=C$  into this chain may overcome this trend. This was supported by the observation that oxidations of *cis*- and *trans*- $\beta$ -methylstyrene each gave two product spots on t.l.c. analysis. Analysis of the products with higher  $R_f$  by  $^1\text{H}$  NMR spectroscopy each showed an  $A_2B_2$  pattern in the aromatic region, indicating that both *cis*- and *trans*- $\beta$ -methylstyrene had been hydroxylated in the para-position. Comparison of the intensities of spots on the t.l.c. showed that the product with the lower  $R_f$  had been formed in larger quantity from the *trans*-isomer than from the *cis*-isomer. There was insufficient product from the oxidation of either isomer for  $^1\text{H}$  NMR spectroscopic analysis, but t.l.c. analysis of the product from *trans*- $\beta$ -methylstyrene showed that it co-chromatographed with *trans*-cinnamyl alcohol.

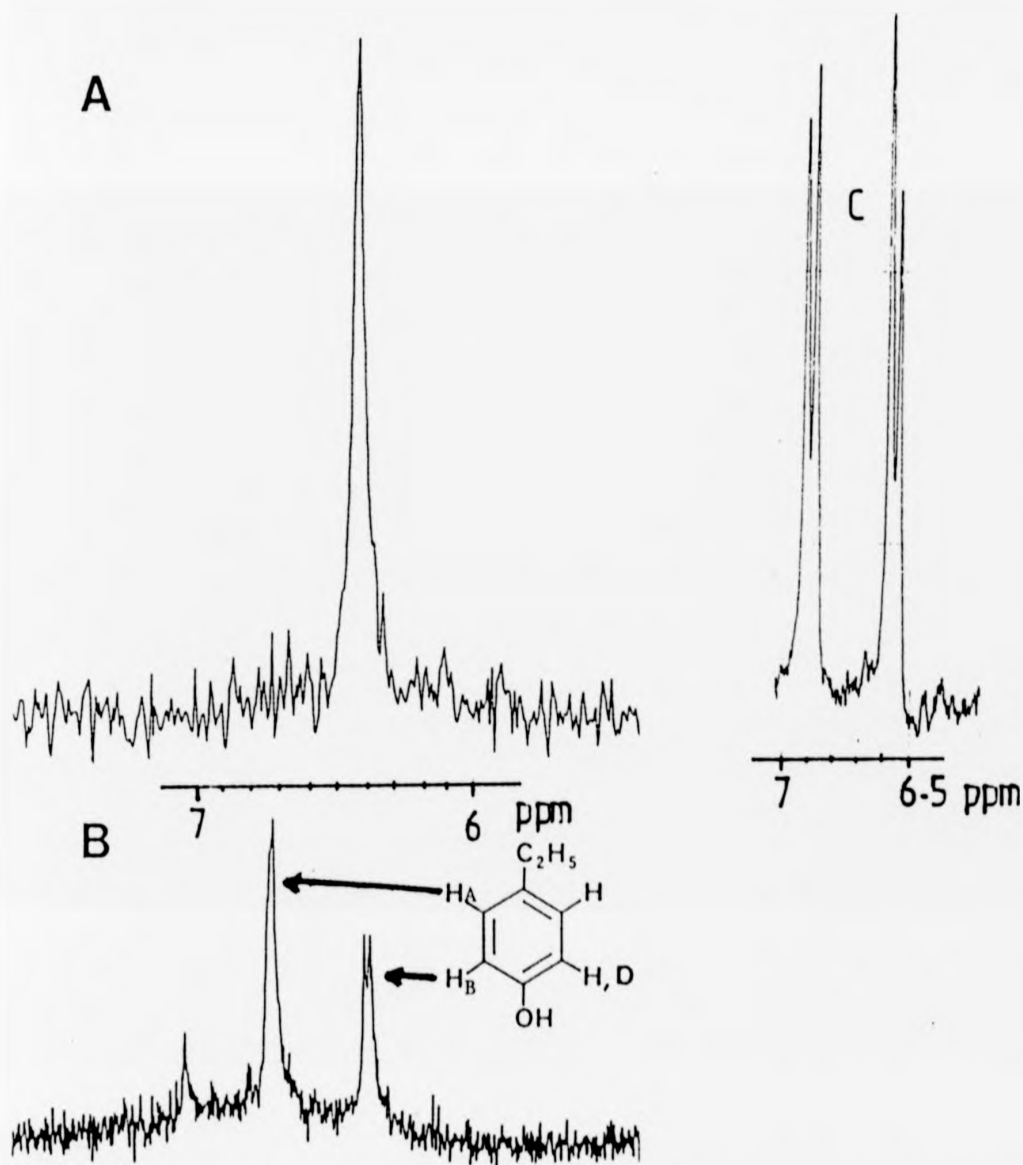
This was the first time that we had observed co-oxidation



of a side-chain and it could be argued that there is an 'electronic' requirement, i.e., oxidation only occurs at those positions where a radical or carbocation can be stabilised by conjugation. With alkyl side-chains longer than  $C_2$  only the carbon  $\alpha$  to the aromatic ring can fulfil this requirement and steric hindrance may make this position unavailable to the oxidant. Assuming that there is only one iso-enzyme in the bacterial system used, this argument fails to account for the known aliphatic oxidations, e.g., of methane<sup>17</sup>. The author will return to this point in Chapter 5.

Frequently aromatic hydroxylations are accompanied by an 'NIH shift' (see introduction to this Chapter) and this does give some indication of the nature of the 'active oxygen' species. For this reason investigations were undertaken to determine if such a rearrangement accompanied oxidations mediated by the M.C. monooxygenase. The substrate initially chosen for this investigation was [4-<sup>2</sup>H]ethylbenzene because ethylbenzene was found to be oxidised relatively efficiently, ca. 50% of the product being 4-ethylphenol. <sup>1</sup>H NMR spectroscopic analysis of the product from [4-<sup>2</sup>H]ethylbenzene after preparative t.l.c. purification, showed differences in the  $A_2B_2$  pattern when compared to authentic 4-ethylphenol (Fig. 3.15 B & C). These differences can be explained by postulating the occurrence of an 'NIH shift'. The decrease in intensity of the higher field doublet is due to partial deuterium retention adjacent to the hydroxyl-bearing carbon (position Hb in Fig. 3.15). With deuterium in this position the adjacent proton (shown as Ha) will not show any coupling and consequently will appear as a singlet (ignoring small <sup>3</sup>J coupling between H and D). This singlet will be superimposed on the doublet from the undeuterated half of the molecule. Support for this interpretation comes from the 61.4 MHz <sup>2</sup>H NMR spectrum (Fig. 3.15 A) of this product. This

Fig. 3.15 A Aromatic region of the 61.4 MHz  $^2\text{H}$  NMR spectrum of the oxidation product of  $[4\text{-}^2\text{H}]$ ethyl benzene  
 B Aromatic region of the 220 MHz  $^1\text{H}$  NMR spectrum of the oxidation product of  $[4\text{-}^2\text{H}]$ ethyl benzene  
 C Aromatic region of the 220 MHz  $^1\text{H}$  NMR spectrum of authentic 4-ethylphenol



spectrum shows a single deuterium peak corresponding in chemical shift to the high field portion of the  $A_2B_2$  resonances in the  $^1H$  NMR spectrum.

Attempts to quantify the deuterium retention by electron impact mass-spectral analysis of both the parent compound and certain derivatives have proved inconclusive. The published value<sup>18</sup> of ca. 60% deuterium retention was based on some of these values and was supported by the integrated  $^1H$  NMR spectrum.

The 'NIH shift' has been reported to occur with certain monooxygenases when the migrating group is chlorine or bromine. However, with the M.C. mono-oxygenase attempts to oxidise 4-bromo-ethylbenzene were unsuccessful.

Preliminary investigations of the oxidation of naphthalene by the M.C. enzyme indicated the formation of 1- and 2-naphthols and a third unidentified product<sup>19</sup>. We confirmed the formation of the naphthols (see below) and suggest that the so-called third product is an artefact, probably a fatty acid extracted from the cells used. Both naphthols were identified by their  $^1H$  NMR spectra and their relative amounts were estimated as 1.6:1 (1-naphthol:2-naphthol) by h.p.l.c. This result contrasts with oxidations of naphthalene mediated by other enzymes in which only a trace of 2-naphthol was seen<sup>9</sup>.

Microsomal oxidation of  $[1-^2H]$ - and  $[2-^2H]$ naphthalene has previously been used in determining the mechanism of the 'NIH shift'<sup>10</sup> (see introductory section to this Chapter). We felt that the use of these substrates with the M.C. enzyme might give some indication of the mechanism that leads to the formation of 2-naphthol.

Jerina and his co-workers<sup>9</sup> showed that the traces of 2-naphthol produced in the microsomal oxidation of naphthalene arose from spontaneous rearrangement of naphthalene 1,2-oxide. No evidence

was found to implicate naphthalene-2,3-oxide as an intermediate in the oxidation pathway. An attempt to synthesise naphthalene-2,3-oxide<sup>9</sup> gave the oxepin (**6**) as the only isolable product. No 2-naphthol was observed and this led Jerina to suspect that the rearrangement shown in Fig. 3.12 was irreversible. If this is true then the relatively large amount of 2-naphthol produced in the M.C. oxidation of naphthalene is unlikely to have arisen *via* naphthalene-2,3-oxide. If there is an epoxide intermediate, as the 'NIH shift' results indicate, then the 2-naphthol produced must be a product of the rearrangement of naphthalene-1,2-oxide.

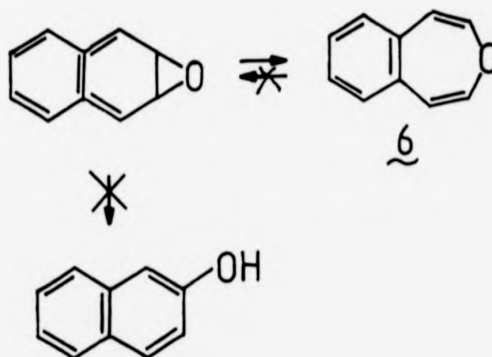


Fig. 3.12

Assuming that the enzymic oxidation of either 1- or 2-deuterated naphthalene to give naphthalene-1,2-oxide proceeds without a significant secondary isotope effect, then this oxidation should occur with equal probability at any one of four equivalent positions, leading to the mixture of intermediates (or products), shown in Fig. 3.12(a).

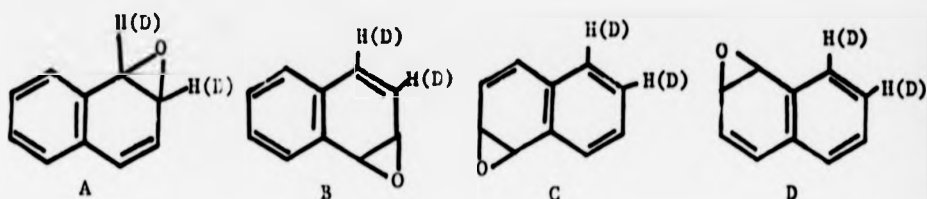


Fig. 3.12(a)

Available evidence suggests that these compounds normally rearrange to a dienone and then lose hydrogen or deuterium to give the product naphthol (see NIH shift, this Chapter). Mass spectral analysis of this product will only show the total amount of deuterium and will not show the occurrence of an NIH shift. N.m.r. spectroscopy, however, can be used to identify the position of the deuterium label in deuterated naphthols. Fig. 3.12(b) shows  $^1\text{H}$  n.m.r. spectra for authentic 1- and 2-naphthols. Because the chemical shift of deuterium will not differ significantly from that of hydrogen,  $^2\text{H}$  n.m.r. spectroscopy can be used to demonstrate that NIH shift has occurred. By utilising the fact that deuterium loss from compounds B, C and D (Fig. 3.12(a)) is unlikely, the areas of resonances from their deuterium atoms can be used as internal standards for the measurement of deuterium at the 2-position (1-naphthol) and 1-position (2-naphthol).

The enzymic oxidation of 1- and 2-deuterated naphthalene was carried out four times, twice with the crude extract utilised for previous oxidations and twice with whole cells. In the second whole cell oxidation potassium formate was added to increase the yield. The average deuterium retention found in these oxidations is shown in Fig. 3.13 (atom % of deuterium values shown). Due to the low conversion of substrate to product and the insensitivity of  $^2\text{H}$  n.m.r. spectroscopy, these values are accurate to approximately  $\pm 10\%$  of the value shown. It is emphasised that on no occasion could any deuterium be detected at the 3-position of 2-naphthol obtained from the oxidation of  $[2\text{-}^2\text{H}]\text{naphthalene}$ .

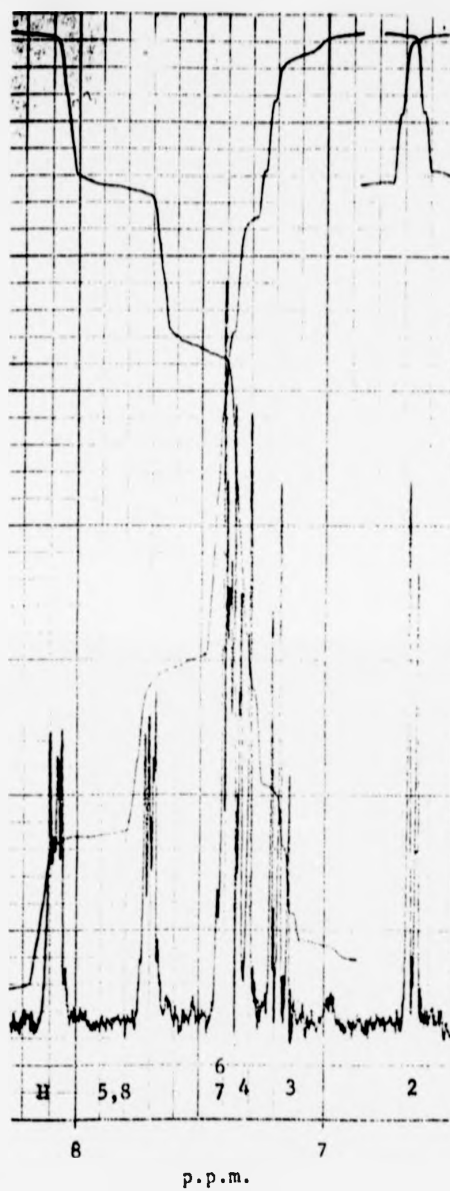
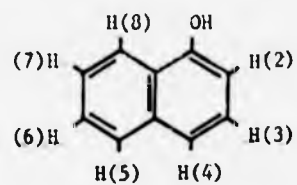


Fig. 3.12(b)  $220\text{ MHz }^1\text{H}$  NMR spectrum of  $\alpha$ -naphthol



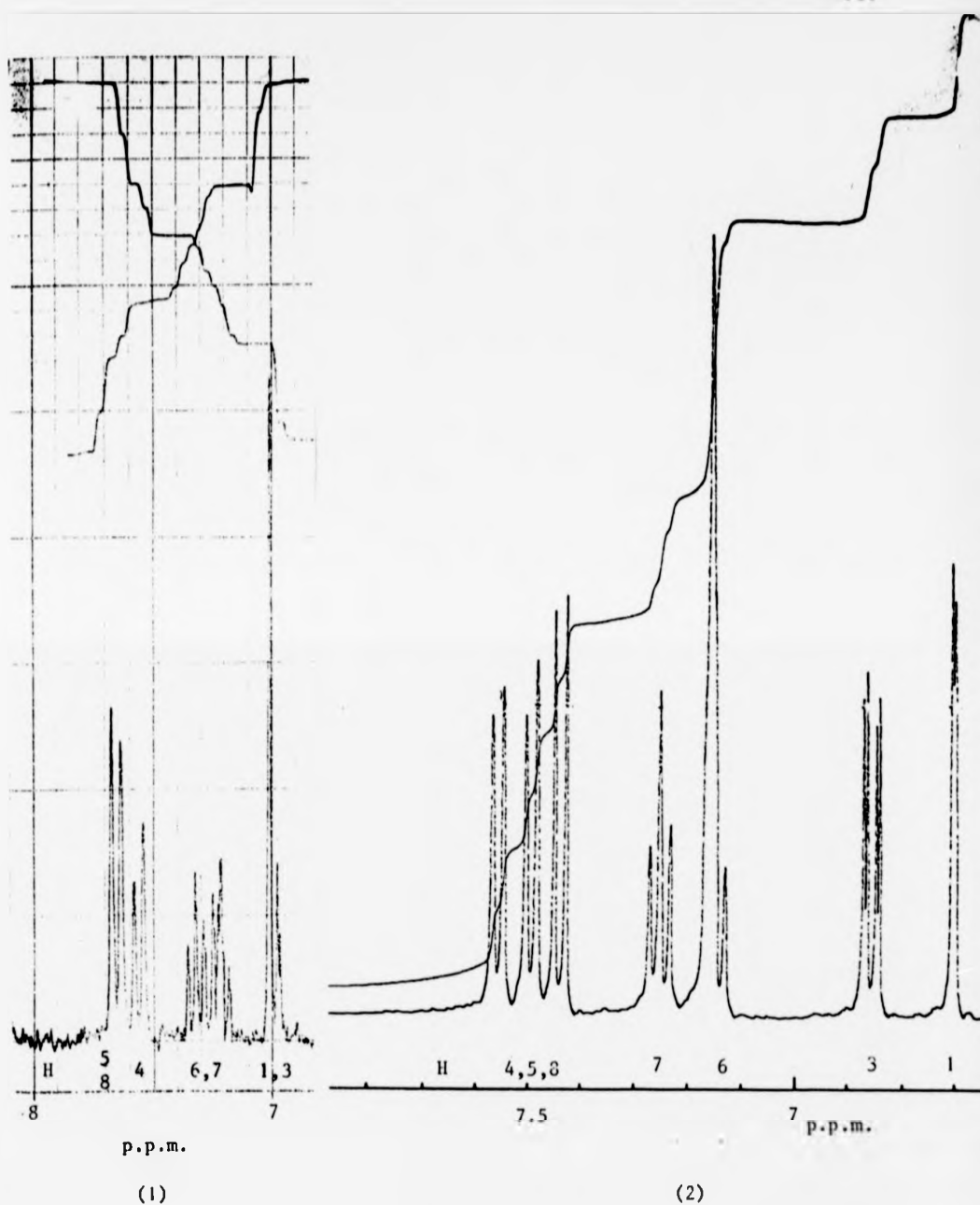
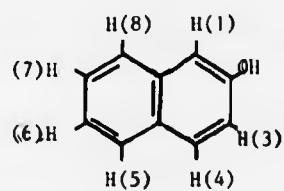


Fig. 3.12(b)

- (1) 220  $^1\text{H}$  NMR spectrum of  $\beta$ -naphthol in  $\text{CCl}_4$
- (2) 440  $^1\text{H}$  NMR spectrum of  $\beta$ -naphthol in benzene



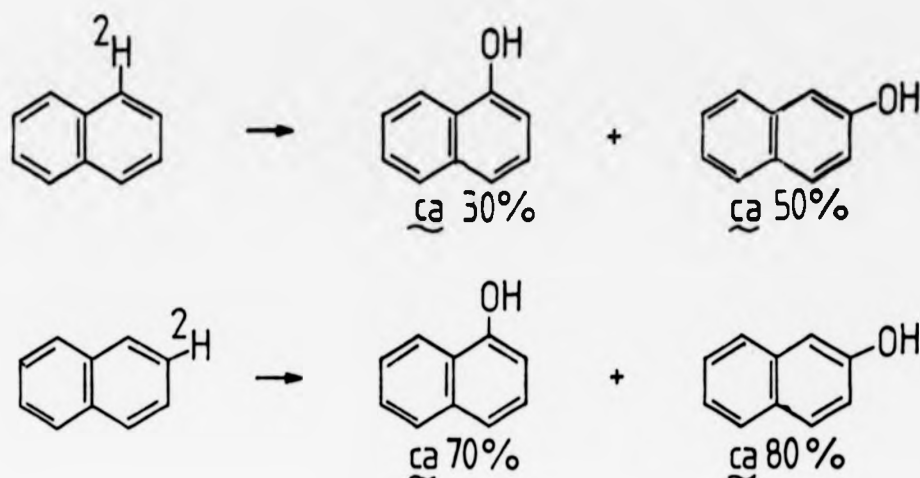


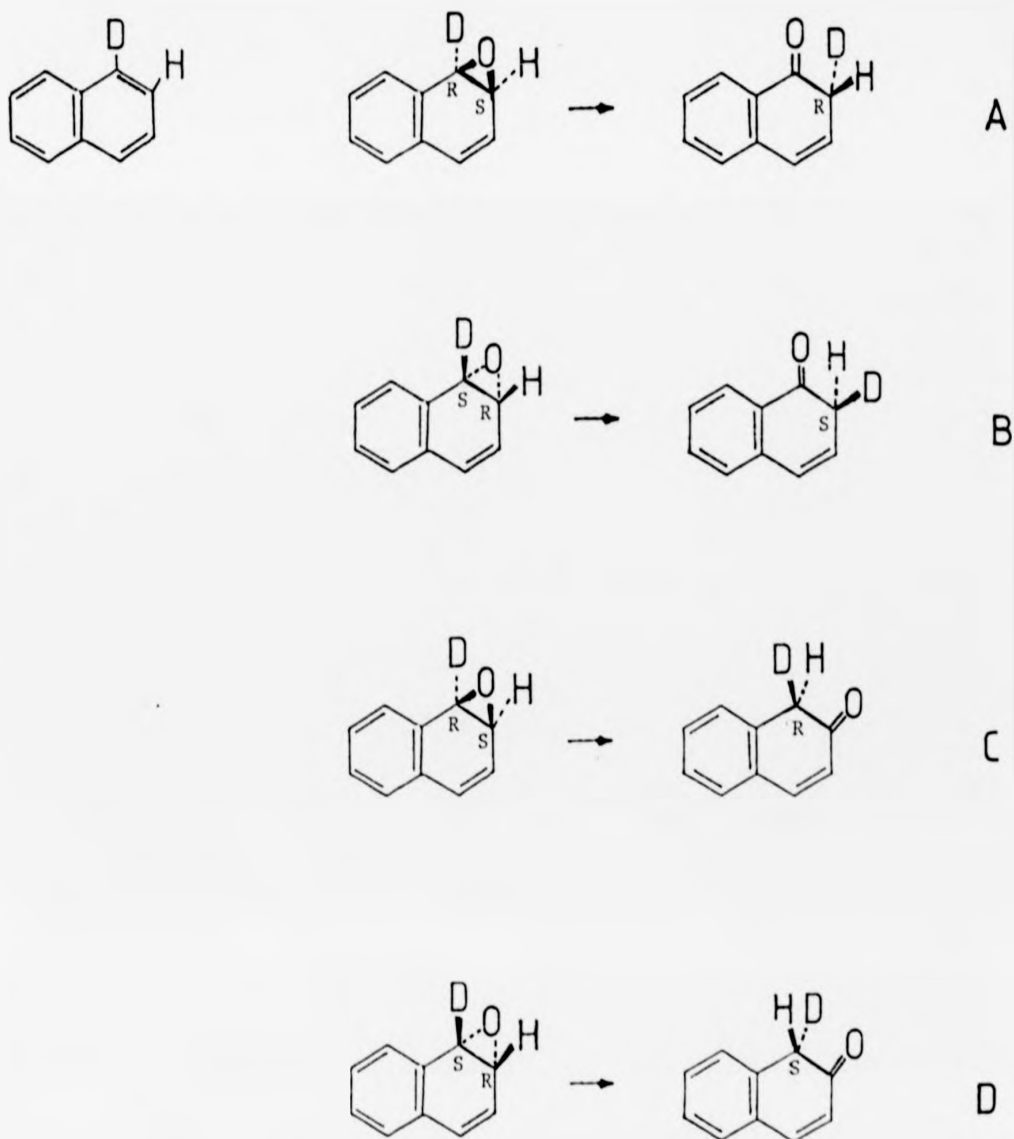
Fig. 3.13

These results, unlike  $\text{P}_{450}$ -dependent oxidations of deuterated naphthalenes, cannot be explained by postulating dissociation of an intermediate epoxide from the enzyme followed by spontaneous isomerisation to the phenol. If this were the case then 2-naphthol should not be formed in such large quantities and the deuterium retention in any particular product should be independent of the deuterium's initial position (see 'NIH shift' section).

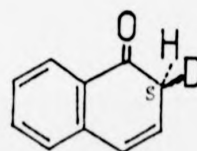
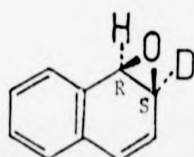
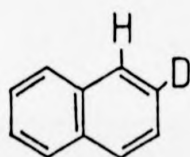
Assuming that naphthalene can only bind in one configuration to the active site of the enzyme then it is reasonable to assume that epoxidation will be stereospecific. If this is so, then Scheme 3.1 shows the possible intermediates that would be produced and their rearrangement products. From this scheme it can be seen that some of the dienone intermediates are equivalent, i.e.  $\text{A} = \text{F}$ ,  $\text{B} = \text{E}$ ,  $\text{C} = \text{H}$  and  $\text{D} = \text{G}$ . If enolisation of a dienone is enzyme-mediated and the proton or deuterium is removed by a basic group on the



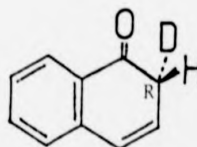
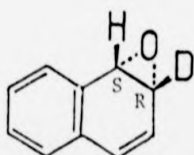
Scheme 3.1 Possible intermediates and products from the enzymic oxidation of deuterated naphthalenes.



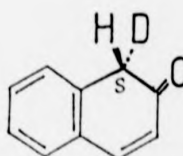
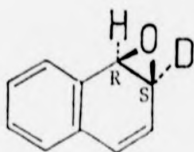
Scheme 3.1 Continued



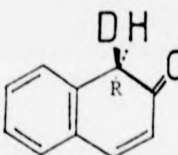
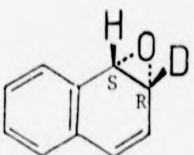
E



F



G



H

enzyme's active site then, depending on the stereochemistry of the dienones, all or none of the deuterium should be removed and the paired groups shown above should retain the same amount of deuterium in the product naphthols.

The deuterium retention values shown in Fig. 3.13 do not appear to support this pathway, but neither are they consistent with enzyme-mediated, stereospecific removal of a proton or deuterium from a racemic dienone, which would lead to *ca.* 50% retention in all cases. The conversions of [1-<sup>2</sup>H]- and [2-<sup>2</sup>H]naphthalene to 1-naphthol show 30% and 70% deuterium retention, respectively. This is consistent with a mechanistic pathway that involves stereospecific removal of either a proton or deuterium from a pure dienone enantiomer. The 30% 'discrepancy' in both cases could be accounted for by some dissociation of the dienone from the active site of the enzyme before the enolisation step, in which case a primary kinetic isotope effect on the spontaneous enolisation could account for the values found.

The data for deuterium retention in the conversion of [1-<sup>2</sup>H] and [2-<sup>2</sup>H]naphthalene to 2-naphthol shows values of 50% and 80% deuterium retention, respectively. This could be taken to indicate weaker binding of the 2-keto intermediate relative to the 1-keto intermediate to the enzyme's active site. This would result in greater loss of intermediate dienone before the enolisation step, followed by spontaneous enolisation, with a primary kinetic isotope effect again partially determining the amount of deuterium retained.

As was mentioned earlier this explanation has assumed that there is only one possible binding configuration of naphthalene to the enzyme's active site. If there were more than one then a mixture of the two possible enantiomeric dienones would be produced. The 30:70% ratio of deuterium retained in the oxidation of [1-<sup>2</sup>H]- and

[2-<sup>2</sup>H]naphthalene to 1-naphthol could then represent the relative binding affinities of these two modes with complete enzyme-mediated proton or deuterium removal. This possibility would still not completely explain the results for 2-naphthol. It would again require some contribution from non-enzymic rearrangement and/or enolisation, due to dissociation of the intermediate from the enzyme.

### 3.5 EXPERIMENTAL

#### 4-Ethylphenol 3,5-dinitrobenzoate

The procedure in Vogel<sup>20</sup> was used. Recrystallisation of the crude product from ethanol gave white-coloured crystals, m.p. 133.5°C (lit. m.p. 132°C)<sup>21</sup> that showed a single spot on t.l.c. (CH<sub>2</sub>Cl<sub>2</sub>, silica gel, u.v.) with R<sub>f</sub> 0.18.

<sup>1</sup>H NMR (CCl<sub>4</sub>, p.p.m.): 1.3 (t, 3 H), 2.7 (q, 2 H), 7.08, 7.22 (A<sub>2</sub>B<sub>2</sub> system, 4 H, J ca. 10 Hz), 9.2 (s, 3 H).

#### 3,5-Dinitrobenzoates of ortho, meta and para-cresols

The procedure in Vogel<sup>20</sup> was used. Recrystallisation of the crude products from ethanol gave white-coloured crystals that showed single spots on t.l.c. (CH<sub>2</sub>Cl<sub>2</sub>, silica gel, u.v.) with all R<sub>f</sub>s 0.13. Their m.p.s were ortho 138°C (lit. m.p. 138°C)<sup>21</sup>, meta 166°C (lit. m.p. 165°C)<sup>21</sup>, para 189°C (lit. m.p. 189°C)<sup>21</sup>.

<sup>1</sup>H NMR - Ortho (CDCl<sub>3</sub>, p.p.m.): 2.1 (s, 3 H), 7.2 (m, 4 H), 9.3 (s, 3 H).

<sup>1</sup>H NMR - Meta (CDCl<sub>3</sub>, p.p.m.): 2.35 (s, 3 H), 7.2 (m, 4 H), 9.3 (s, 3 H).

$^1\text{H}$  NMR - Para ( $\text{CDCl}_3$ , p.p.m.): 2.4 (s, 3 H), 7.1, 7.25 ( $\text{A}_2\text{B}_2$  system, 4 H,  $J$  ca. 9 Hz), 9.3 (s, 3 H).

Rac-1-Phenylethanol 3,5-dinitrobenzoate

This was prepared by the method of Vogel<sup>20</sup>. Recrystallisation of the crude product from methanol gave white-coloured crystals, m.p.  $90^\circ\text{C}$  (lit. m.p.  $94^\circ\text{C}$ )<sup>21</sup>, t.l.c. ( $\text{CH}_2\text{Cl}_2$ , silica gel, u.v.) showed a single spot  $R_f$  0.18.

$^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.): 1.75 (d, 3 H), 6.1 (q, 1 H), 7.35 (m, 5 H), 9.0 (s, 3 H).

4-Ethylphenol  $\alpha$ -naphthylurethane

The standard method from Vogel<sup>20</sup> was used. Recrystallisation of the crude product from petrol ether (b.p.  $80\text{--}100^\circ\text{C}$ ) gave white crystals, m.p.  $130^\circ\text{C}$  (lit. m.p.  $128^\circ\text{C}$ )<sup>21</sup>; the electron impact mass spectrum showed a weak molecular ion at  $m/z$  291.

$^1\text{H}$  NMR ( $\text{CDCl}_3$ , p.p.m.): 1.25 (d, 3 H), 2.65 (q, 2 H), 7.0–8.0 (complex m, 11 H).

4-Ethylanisole<sup>20</sup>

To an ice-cooled, round-bottom flask fitted with reflux condenser and containing a stirrer bar was added 4-ethylphenol (6.1 g,  $50 \times 10^{-3}$  mol) and aqueous sodium hydroxide solution ( $20 \text{ cm}^3$ , 50% w/v). To the resulting stirred solution was added dimethyl sulphate (6.3 g,  $50 \times 10^{-3}$  mol) dropwise over a 20 minute period. Stirring was continued for a further 40 minutes. The

mixture was then heated at reflux for 2 h, allowed to cool and water ( $20\text{ cm}^3$ ) was added. The aqueous layer was separated, extracted with diethyl ether ( $3 \times 10\text{ cm}^3$ ) and the combined organic fractions were washed with water ( $10\text{ cm}^3$ ), followed by dilute  $\text{H}_2\text{SO}_4$  ( $2 \times 10\text{ cm}^3$ ) and water, until the washings were neutral to litmus paper. The mixture was then dried ( $\text{MgSO}_4$ ), filtered and the ether was removed by flash distillation. Reduced pressure distillation of the residue ( $17\text{ mmHg}$ ,  $87\text{--}88^\circ\text{C}$ ) gave  $2.46\text{ g}$  ( $18 \times 10^{-3}\text{ mol}$ ,  $36.2\%$  yield) of a colourless liquid that on t.l.c. ( $\text{CH}_2\text{Cl}_2$ , silica gel, u.v.) showed a single spot of  $R_f$  0.61. Electron impact mass spectrometry showed a weak molecular ion at  $m/z$  136 and an intense ion at  $m/z$  121.  $^1\text{H NMR}$  ( $\text{CCl}_4$ , p.p.m.): 1.15 (t, 3 H), 2.5 (q, 2 H), 3.7 (s, 3 H), 6.65, 6.95 ( $A_2B_2$  system, 4 H,  $J_{aa}$  7 Hz).

#### Diazomethane

The apparatus was set up as described in Vogel<sup>20</sup> with all of the ground glass joints protected by Teflon sleeves. To the flask was added potassium hydroxide solution (1.67 g in  $2.5\text{ cm}^3\text{ H}_2\text{O}$ ) and absolute ethanol ( $8.3\text{ cm}^3$ ). The mixture was gently stirred and heated at  $60\text{--}62^\circ\text{C}$  whilst a solution of p-tolylsulphonyl-methylnitrosamide (Diazald,  $7.17\text{ g}$ ,  $33.5 \times 10^{-3}\text{ mol}$ ) in diethyl ether ( $42\text{ cm}^3$ ) was added dropwise over  $1\frac{1}{4}\text{ h}$ . A golden coloured liquid distilled over and after all of the Diazald had been added diethyl ether was added dropwise until the distillate became colourless. The diazomethane was protected from light and stored at  $-20^\circ\text{C}$ .

1-Methoxy-1-phenylethane

To a suspension of sodium hydride ( $38.7 \times 10^{-3}$  g,  $1.6 \times 10^{-3}$  mol) in dimethyl sulphoxide ( $1 \text{ cm}^3$ ) was added *rac*-1-phenylethanol ( $12.2 \times 10^{-3}$  g,  $0.1 \times 10^{-3}$  mol) and methyl iodide ( $125 \times 10^{-3} \text{ cm}^3$ ,  $2 \times 10^{-3}$  mol). The resulting solution was stirred at room temperature overnight and then water ( $10 \text{ cm}^3$ ) was added dropwise and the mixture was extracted with diethyl ether ( $3 \times 10 \text{ cm}^3$ ). The ether fractions were combined, washed with brine ( $10 \text{ cm}^3$ ), dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator to produce a colourless oil pure by t.l.c. ( $\text{CH}_2\text{Cl}_2$ , silica gel, u.v.)  $R_f$  0.42. Electron collision mass spectral analysis of this product gave a mass peak at  $m/z$  136.

$^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.): 1.35 (d, 3 H), 3.1 (s, 3 H), 4.2 (q, 1 H), 7.2 (s, 5 H).

Cinnamyl alcohol

This was prepared by reducing cinnamylaldehyde (1.77 g,  $13.4 \times 10^{-3}$  mol) with sodium borohydride in alkaline methanol. The crude product was purified by Kugelröhr distillation (20 mmHg,  $165^\circ\text{C}$ ) giving 1.15 g ( $8.5 \times 10^{-3}$  mol, 63.8% yield) of a clear liquid that crystallised on standing.

$^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.): 3.35 (broad s, 1 H), 4.2 (d, 2 H), 6.2 (doublet of triplets, 1 H), 6.5 (d, 1 H,  $J$  18 Hz), 7.2 (m, 5 H).

[4- $^2\text{H}$ ]Ethylbenzene

To magnesium turnings (1 g,  $42 \times 10^{-3}$  mol) covered by

dry diethyl ether ( $20 \text{ cm}^3$ ) was added a few drops of ethyl iodide. When the ether started to boil under reflux 4-bromoethylbenzene ( $2.1 \text{ g}$ ,  $11 \times 10^{-3} \text{ mol}$ ) was added. When the formation of Grignard reagent was complete  $^2\text{H}_2\text{O}$  ( $1.5 \text{ cm}^3$ , 99.7 atom %) was added and the mixture was heated for *ca.* 5 minutes, cooled on ice and diethyl ether ( $20 \text{ cm}^3$ ) and dilute HCl ( $25 \text{ cm}^3$ ) were added. The ether layer was removed and dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator. Kugelröhr distillation at  $136^\circ\text{C}$  gave  $0.54 \text{ g}$  ( $5.1 \times 10^{-3} \text{ mol}$ , 46.4% yield) of a colourless liquid that showed a single peak on g.l.c. (Apiezon on Chromosorb 101,  $155^\circ\text{C}$ ).  
 $^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.): 1.2 (t, 3 H), 2.55 (q, 2 H), 7.08, 7.18 ( $\text{A}_2\text{B}_2$  system, 4.1 H J *ca.* 7 Hz), [the integrals indicated *ca.* 90%  $^2\text{H}$  incorporation].

#### [1- $^2\text{H}$ ]Naphthalene

To magnesium turnings ( $1 \text{ g}$ ,  $42 \times 10^{-3} \text{ mol}$ ) covered by dry diethyl ether ( $20 \text{ cm}^3$ ) was added a few drops of ethyl iodide. When the ether began to boil under reflux 1-bromonaphthalene ( $4.2 \text{ g}$ ,  $20 \times 10^{-3} \text{ mol}$ ) was added. When the formation of Grignard reagent was complete  $^2\text{H}_2\text{O}$  ( $2.5 \text{ cm}^3$ , 99.7 atom %) was added and the mixture was heated for *ca.* 5 minutes, cooled on ice and diethyl ether ( $20 \text{ cm}^3$ ) and dilute HCl ( $25 \text{ cm}^3$ ) were added. The ether phase was removed and dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator. The resulting crystals were taken up in the minimum of ethyl acetate and purified by passage down a short alumina column using petrol ether (b.p.  $40\text{--}60^\circ\text{C}$ ) as eluant. The solvent was removed by rotary evaporator and the product was recrystallised from ethanol. Finally, sublimation (22 mmHg, oil bath temp.  $75^\circ\text{C}$ ) gave white crystals, m.p.  $81^\circ\text{C}$  (*cf.*



naphthalene m.p. 80°C).

$^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.): 7.35 (m, 4 H), 7.7 (m, 3.1 H) [the integrals indicated ca. 90%  $^2\text{H}$  incorporation].

[2- $^2\text{H}$ ]Naphthalene

This was prepared from 2-bromonaphthalene in the manner described for [1- $^2\text{H}$ ]naphthalene.

$^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.): 7.4 (m, 3.3 H), 7.7 (m, 4 H) [the integrals indicated ca. 70%  $^2\text{H}$  incorporation].

Methylation of the products from enzymic oxidation of [4- $^2\text{H}$ ]ethylbenzene

The oxidation was carried out and products were extracted by the methods outlined in the Materials and Methods section. The solvent was removed by rotary evaporator and to the residue was added dry DMSO (1  $\text{cm}^3$ ), NaH ( $9.84 \times 10^{-3}$  g,  $0.41 \times 10^{-3}$  mol) and MeI ( $58.2 \times 10^{-3}$  g,  $0.41 \times 10^{-3}$  mol). The mixture was stirred overnight at room temperature and then water (10  $\text{cm}^3$ ) was added dropwise. The mixture was then extracted with dichloromethane (3 x 10  $\text{cm}^3$ ) and the combined organic fractions were extracted with brine (10  $\text{cm}^3$ ), dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator. The product was purified by preparative t.l.c. ( $\text{CH}_2\text{Cl}_2$ , silica gel, u.v.).

It is unlikely that any deuterium is lost in this procedure because exchange requires a proton source, which is absent under these basic aprotic reaction conditions.

T.l.c. Characteristics of Products from Enzyme Oxidations

Substrate	Product $R_f$	Solvent
$\alpha$ -Methylstyrene	0.33	$\text{CH}_2\text{Cl}_2$
Ethylbenzene	0.25, 0.14	20% ethylacetate in hexane
Toluene	0.67, 0.13	$\text{Et}_2\text{O}$
Styrene	0.34, 0.15	$\text{CH}_2\text{Cl}_2$
Propylbenzene	0.20	$\text{CH}_2\text{Cl}_2$
Butylbenzene	0.18	$\text{CH}_2\text{Cl}_2$
2-Propylbenzene	0.16	$\text{CH}_2\text{Cl}_2$
$\alpha\beta$ - $\beta$ -Methylstyrene	0.23, 0.14	$\text{CH}_2\text{Cl}_2$
	0.26, 0.13	20% ethylacetate in petrol ether (b.p. 60-80°C)
<i>trans</i> - $\beta$ -Methylstyrene	0.23, 0.14	$\text{CH}_2\text{Cl}_2$
	0.26, 0.13	20% ethylacetate in petrol ether (b.p. 60-80°C)
Naphthalene	0.25, 0.18	$\text{CH}_2\text{Cl}_2$

## CHAPTER 3 - REFERENCES

1. 'Advanced Organic Chemistry', 2nd Edition, J. March, M'Graw-Hill, and references cited therein
2. F. Minisci, *Synthesis*, 1973, 1
3. M. K. Eberhardt, *J. Am. Chem. Soc.*, 1981, 103, 3876
4. N. C. Deno, E. J. Gladfelter and D. G. Pohl, *J. Org. Chem.*, 1979, 44, 3728
5. N. C. Deno, *et al.*, *Tetrahedron*, 1977, 33, 2503
6. G. A. Olah, G. K. Surya Prakash and J. Sommer, *Science (Washington, D.C.)*, 1979, 206, 13
7. G. A. Olah, *Pure and Appl. Chem.*, 1981, 53, 201
8. G. Guroff, *et al.*, *Science (Washington, D.C.)*, 1967, 157, 1524 and references cited therein
9. J. W. Daley, D. M. Jerina and B. Witkop, *Experientia*, 1972, 28, 1129 and references cited therein
10. D. R. Boyd, J. W. Daley and D. M. Jerina, *Biochemistry*, 1972, 11, 1961
11. D. M. Jerina, *J. Am. Chem. Soc.*, 1968, 90, 6525
12. D. M. Jerina, *et al.*, *Biochemistry*, 1970, 9, 147
13. C. E. Carniglia and S. A. Crow, *Arch. Microbiol.*, 1981, 129, 9
14. 'Stereochemistry and its Application in Biochemistry', W. L. Alworth, Wiley-Interscience, and references cited therein
15. D. J. Reed, J. Vimmerstadt, D. M. Jerina and J. W. Daly, *Arch. Biochem. Biophys.*, 1973, 154, 642
16. A. K. Wong, M.Sc. Thesis, University of Warwick
17. D. Stirling, J. Colby and H. Dalton, *Biochem. J.*, 1979, 177, 361
18. H. Dalton, *et al.*, *J. Chem. Soc. Chem. Commun.*, 1981, 482
19. H. Dalton, personal communication
20. 'Textbook of Practical Organic Chemistry', 4th Edition, Vogel, 1978, Longman
21. 'CRC Handbook of Tables for Organic Compound Identification', 3rd Edition, CRC Press

## CHAPTER 4

### ALKENES AND ALKYNES

#### 4.1 OBJECTIVES

There were two main reasons for undertaking the work described in this Chapter. Epoxidation of olefinic groups by the monooxygenase of M.C. had already been shown to occur<sup>1</sup>. Any stereochemical changes arising as a result of this epoxidation should give some indication of the nature of the oxidant and the mechanism utilised.

The second line of work arose as a result of the inhibition of the enzymic activity by certain alkyne groups<sup>2</sup>. It was hoped that this inhibition might be used for isolation and purification of the enzyme.

#### 4.2 ALKENE EPOXIDATIONS

Of all of the different types of reaction catalysed by the various monooxygenase enzymes, olefin epoxidation is probably the easiest to achieve chemically. The standard laboratory method of epoxidation is the *Prilezhacv reaction* in which a peracid such as perbenzoic acid reacts with an olefin at room temperature to produce the epoxide. Two mechanisms have been advanced that could explain the experimental findings, and these are shown in Figs. 4.1 and 4.2. The first, shown in Fig. 4.1, utilises an 'oxenoid' mechanism (see models section of Chapter 1) involving the reaction of an electron deficient oxygen atom with the olefin<sup>3</sup>. Experimental evidence that

supports this proposal includes the observation that electron donating groups on the olefin and electron withdrawing groups on the peracid (e.g. trifluoroperacetic acid) both increase the rate of reaction<sup>3</sup>. Other experimental observations consistent with this mechanism include the retention of stereochemistry, i.e. *trans*-olefins give *trans*-epoxides<sup>3</sup>. The alternative mechanism, shown in Fig. 4.2, involves a concerted 1,3-dipolar interaction of a peracid tautomer with the olefin to give a five membered ring that rearranges, as shown, to give the product epoxide. This mechanism is also consistent with the experimental observations mentioned above but certain kinetic data are inconsistent with this mechanism<sup>3</sup>.

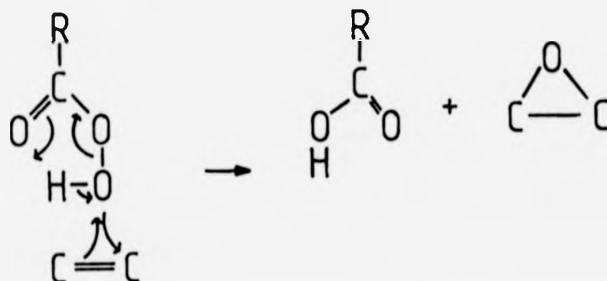


Fig. 4.1

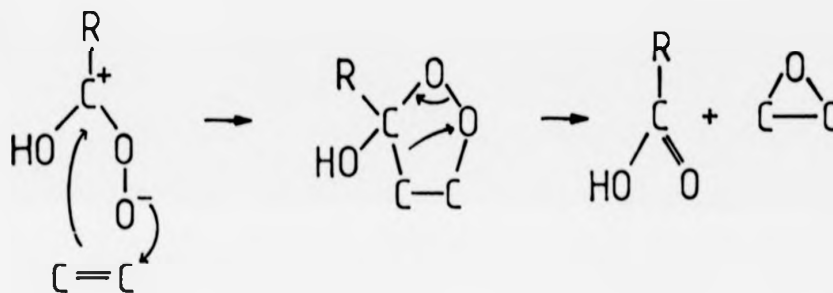
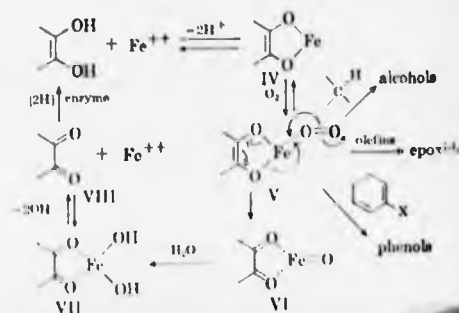


Fig. 4.2

The two monooxygenase model systems, Fenton's reagent and the Udenfriend system (see models section in Chapter 1), are both able to epoxidise olefins<sup>4</sup>. In aqueous solutions Fenton's reagent is believed to give rise to hydroxyl radicals that effect the observed oxidations (see models section in Chapter 1). Due to the nature of this oxidant reactions involving Fenton's reagent usually give a mixture of products. Thus, oxidation of an olefin, e.g. cyclohexene<sup>4</sup>, results in the formation of several by-products in addition to the epoxide. As cyclohexene epoxide is the only product obtained during oxidations of cyclohexene by the Udenfriend system<sup>4</sup>, it seems unlikely that a hydroxyl radical is involved in this case. The use of catalase and the inability of hydrogen peroxide to replace dioxygen in this system suggests that the oxidant is not at the peroxide oxidation level<sup>4</sup>. These results have led Hamilton and his co-workers<sup>5</sup> to propose the mechanism shown in Fig. 4.3 for the production of the 'active oxygen' species in the Udenfriend model system.

A number of other transition metal complexes are known to act as epoxidising agents, e.g. the chromyl-oxo complex described by Sharpless<sup>6</sup>. Transition metal ions such as vanadium, molybdenum, chromium or tungsten in the presence of a peroxide such as *t*-butylhydroperoxide have also been used to epoxidise olefins<sup>7</sup>. These metals, however, cannot be replaced by metals such as Fe<sup>II</sup> and Co<sup>II</sup>, i.e. those that induce free radical decomposition of the hydroperoxides<sup>7</sup>. Epoxidations with the transition metal complexes such as vanadium all proceed with the same *syn*-stereospecificity as observed with the peracids<sup>8</sup>.

Fig. 4.3 Reproduced from G. A. Hamilton, *et al.*, *J. Am. Chem. Soc.*, 1964, 86, 3391



## 4.3

ENZYMIC OXIDATION OF ALKENES

It was hoped that oxidation of an allylic methyl group could be used to determine if a radical or direct insertion mechanism were used by the M.C. monooxygenase. Abstraction of a hydrogen atom from such a methyl group would generate the symmetrical intermediate shown in Fig. 4.4. Oxygen addition could then take place at either of the two equivalent carbons. If one of these two positions were labelled with  $^{13}\text{C}$  then NMR spectroscopy could be used to show whether this label had become equilibrated between the two positions, see Fig. 4.4. If a direct insertion were used then no scrambling of the label would occur. To this end  $\alpha$ -methylstyrene was oxidised, but as already reported (Chapter 3, arenes), oxidation occurred exclusively on the aromatic ring. According to the TLC analysis, neither 2-methylbut-1-ene nor 2-methylbut-2-ene, showed any evidence of oxidation by the M.C. enzyme. Both cyclohexene and

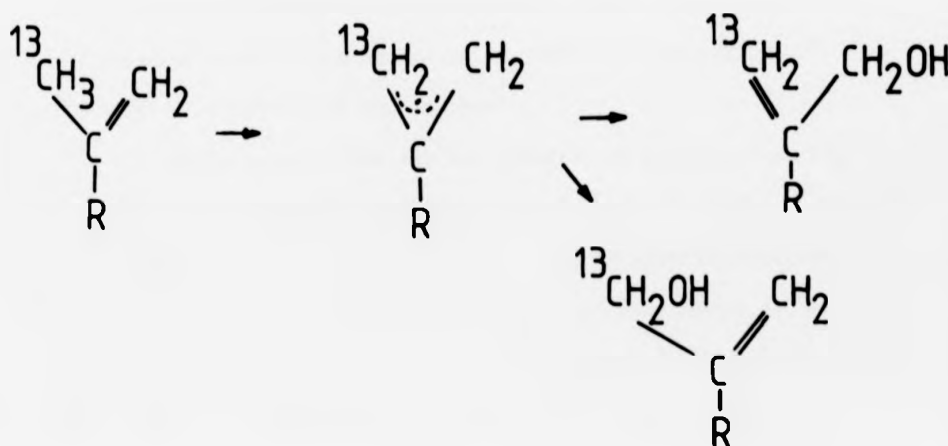


Fig. 4.4

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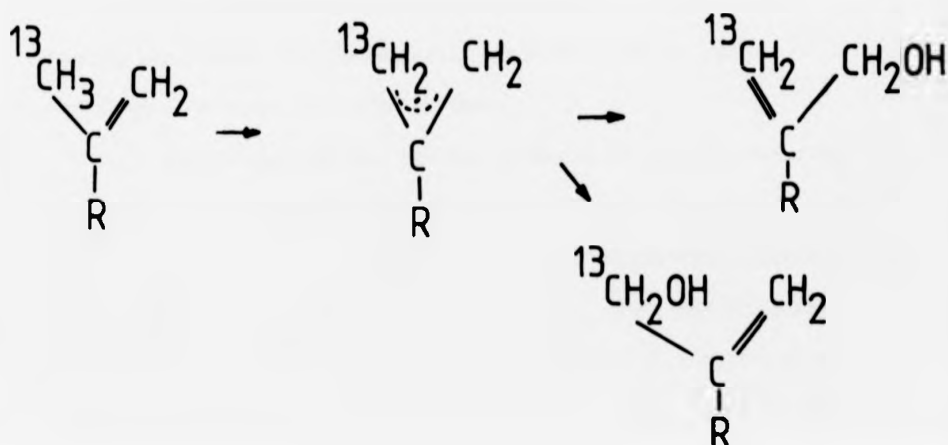


Fig. 4.4



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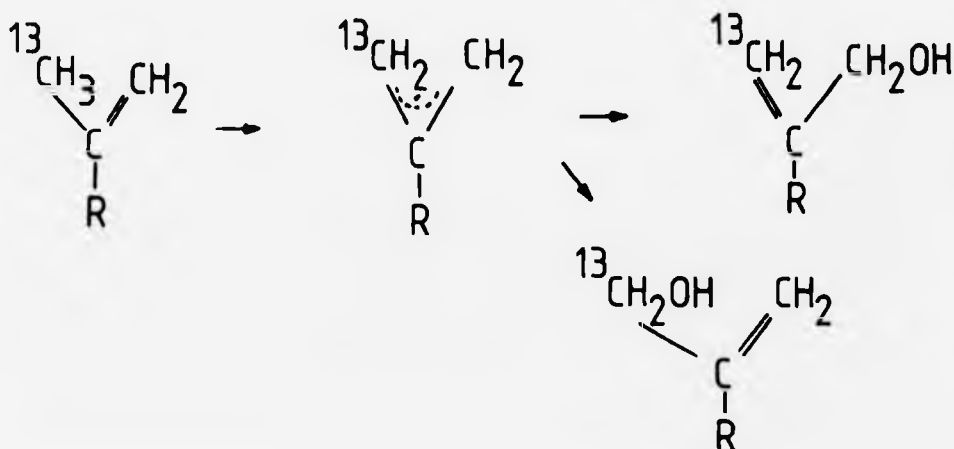


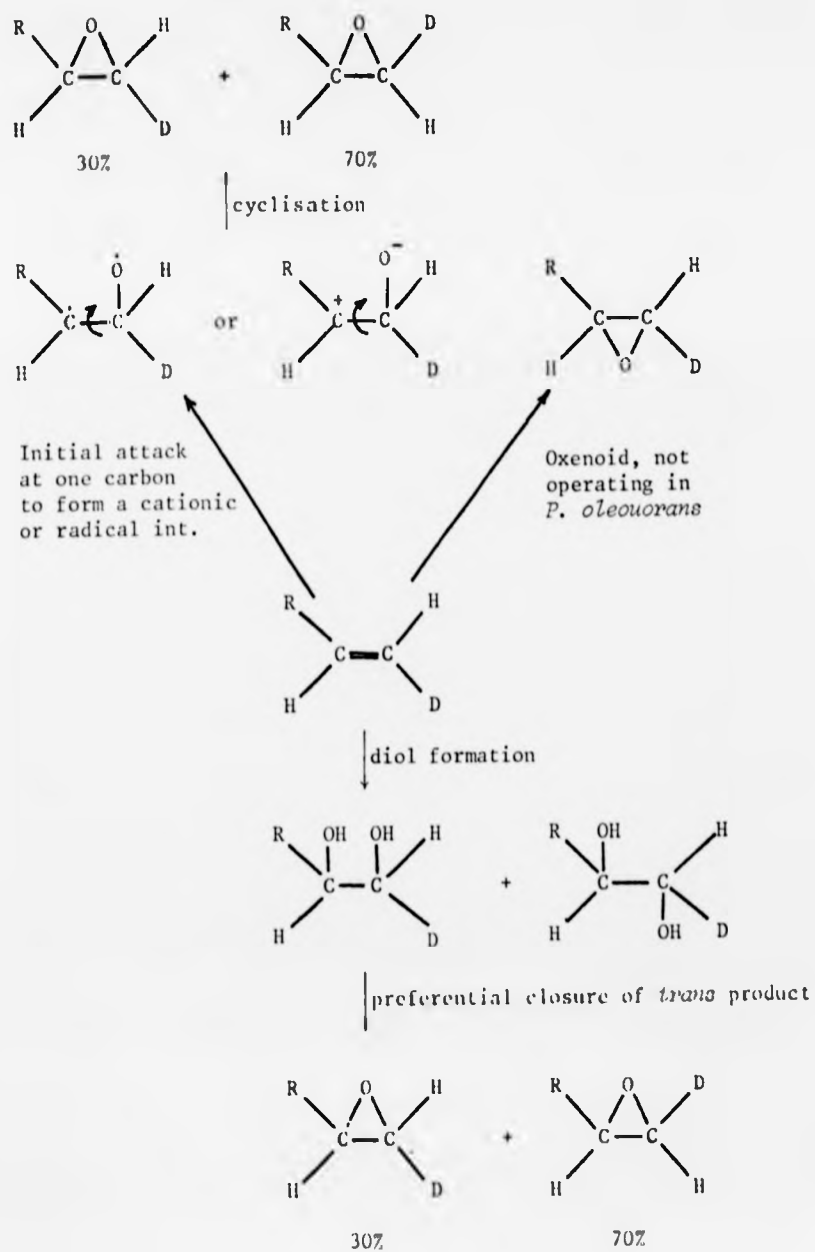
Fig. 4.4

1-methylcyclohexene were oxidised by the M.C. monooxygenase. Preliminary results indicated that cyclohexene may have been oxidised to cyclohex-2-en-1-ol. However, larger scale oxidations, using both substrates, failed to give sufficient material for  $^1\text{H}$  NMR spectroscopic analysis. The fact that the oxidations were highly inefficient, together with the  $\alpha$ -methylstyrene result, suggested to us that the allylic region of these compounds is in general a poor substrate for the M.C. monooxygenase enzyme. This may be due to the reasons suggested in Chapter 3, i.e. steric hindrance of the allylic methyl group preventing the substrate from reaching the oxidant efficiently. This does not, however, explain why the unhindered end of the acyclic methylbutenes was not oxidised, particularly as both *cis* and *trans*-but-2-ene and the phenyl ring of  $\alpha$ -methylstyrene are oxidised<sup>1</sup>. The low yields obtained in the oxidation of the two cyclic olefins were also unexpected, cyclohexane itself is oxidised relatively efficiently<sup>1</sup> so this cannot be due to the non-planar nature of the substrate. The obvious inefficiency found for the oxidation of these types of compound caused us to abandon this line of work.

Olefin epoxidation, whether chemical or enzymic, normally proceeds with retention of configuration. However, May and co-workers<sup>8</sup> found that epoxidation (*cf.* Scheme 4.2) by *Pseudomonas oleovorans* gave a product with 70% inversion of geometry. This result is inconsistent with an 'oxenoid' mechanism and it was of interest to see if such a result were obtained during epoxidations using the M.C. monooxygenase. Colby and his co-workers<sup>1</sup> had shown that propylene oxide was the only product obtained from the oxidation of propene by the monooxygenase from M.C. The oxidation of *cis*-[1- $^2\text{H}$ ]propene by this enzyme was carried out and the product was extracted into benzene

## Scheme 4.2

Possible explanations for May's results of olefin epoxidation with *P. oleovorans*

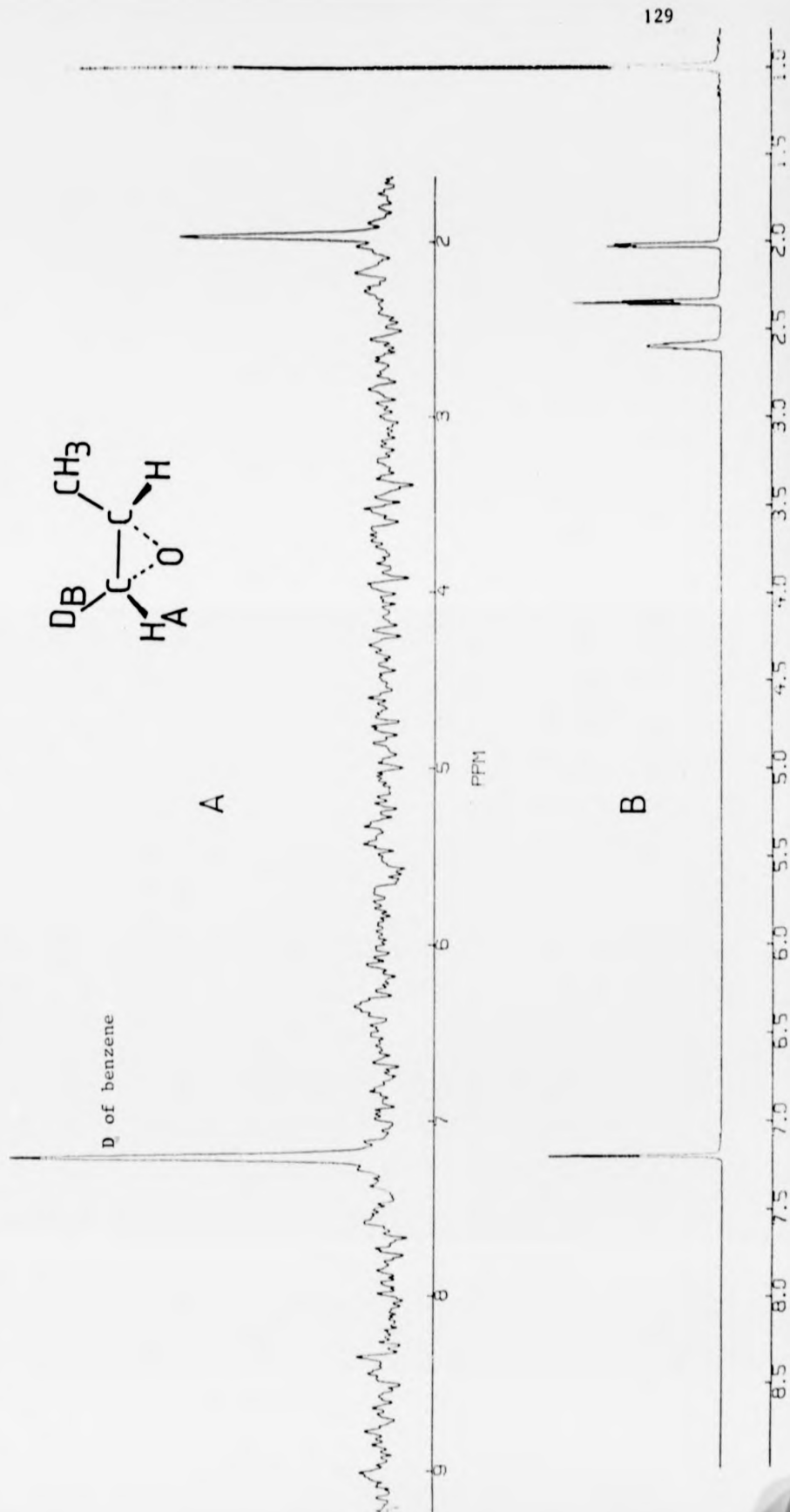


and analysed by both proton and deuterium NMR spectroscopy. The  $^2\text{H}$  NMR spectrum obtained is shown in Fig. 4.5 along with a  $^1\text{H}$  NMR spectrum of authentic propylene oxide. The small chemical shift difference between the spectra can be explained by an isotope effect. The  $^1\text{H}$  NMR spectrum of propylene oxide has been assigned by Khanh<sup>9</sup> who showed that the broad multiplet at 2.6 p.p.m. was due to the methine proton; the triplet at 2.35 p.p.m. was due to the methylene proton, shown as  $\text{H}_\text{A}$  in Fig. 4.5, and the multiplet at 2.2 p.p.m. was due to  $\text{H}_\text{B}$ . The  $^1\text{H}$  NMR spectrum obtained from the product of the oxidation of *cis*-[1- $^2\text{H}$ ]propene by the M.C. monooxygenase was the same as that obtained from authentic propylene oxide except that the multiplet at 2.2 p.p.m. was absent. As the  $^2\text{H}$  NMR spectrum shows there is no peak around 2.3 p.p.m., it can be concluded that oxidation has occurred in a *syn*-stereospecific manner and any product arising from rotation around the C-C bond accounts for less than 10% of the resultant product. This result is consistent with an oxenoid mechanism. It has previously been shown<sup>10</sup> that oxidation of propene by the M.C. monooxygenase gives a racemic mixture of products, i.e. both the S and R epimers are produced and consequently this enzyme does not differentiate between the *re* and *si* faces in binding the olefinic substrate.

#### 4.4 INHIBITORY PROPERTIES OF N-PROPARGYLVALERAMIDE

In oxidations utilising the M.C. monooxygenase acetylenic compounds can be used as enzymic inhibitors, whereas these compounds would be oxidised by cytochrome  $\text{P}_{450}$ <sup>11</sup>. The possibility that the M.C. monooxygenase may also be accepting such acetylenic compounds as substrates and that a very slow turnover may result in apparent

Fig. 4.5 A 61.4 MHz  $^2\text{H}$  NMR spectrum of the product of enzymic oxidation of *cis*[1- $^2\text{H}$ ]propene  
 B 220 MHz  $^1\text{H}$  NMR spectrum of authentic propylene oxide  
 (Solvent: benzene)

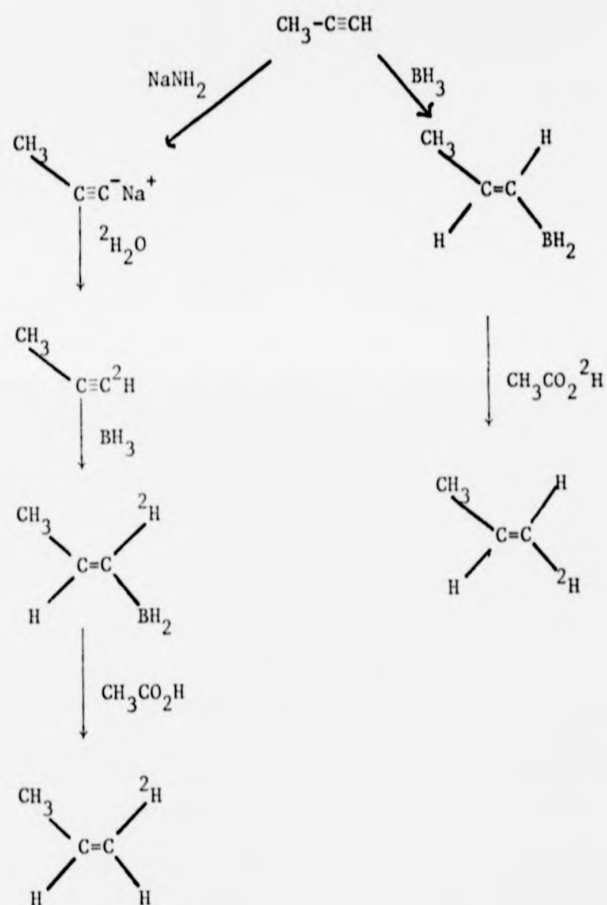


inhibition was suggested to us<sup>12</sup>. If this were the case then it offered the possibility of utilising an acetylenic group in affinity chromatography to purify the enzyme. As an initial model we decided to couple a propargyl unit to a short hydrocarbon chain *via* an amide bond and to include this compound in an oxidation of  $\alpha$ -methylstyrene to see if there was any inhibition of this oxidation. The oxidations were carried out using concentrations of the potential inhibitor of between  $1 \times 10^{-5}$  and  $5 \times 10^{-3}$  molar and the amount of product formed was estimated by GLC (20% DEGS, 120°C) and by its u.v. absorption at 256 nm. Both of these methods showed a maximum inhibition (*ca.* 50%) at around  $2.5 \times 10^{-3}$  molar. If the propargyl unit were competing with the  $\alpha$ -methylstyrene for binding to the enzyme's active site, then increasing the concentration of the inhibitor should have decreased the amount of product from the oxidation of  $\alpha$ -methylstyrene. As this was not the case, and the reason for this behaviour was not apparent, then obviously we could not utilise the propargyl side chain as we had hoped in the enzyme purification.

#### 4.5 SYNTHESIS OF DEUTERATED PROPENES

To the sodium salt of propyne, produced from propyne and sodamide, was added  $2\text{H}_2\text{O}$  to give  $[1-^2\text{H}]$ propyne with 83% deuterium incorporation. Reduction of  $[1-^2\text{H}]$ propyne using acidic  $\text{Cr}^{\text{II}}$  to give *trans*- $[1-^2\text{H}]$ propene<sup>13</sup>, took *ca.* six days during which the reduction was monitored by GLC (Chromosorb 101, 90°C).  $^1\text{H}$  NMR spectroscopic analysis of the product showed it to contain effectively no deuterium so the reaction was repeated and monitored by  $^1\text{H}$  NMR spectroscopy. The results from this study suggested that deuterium was not being lost from the propyne, but was being lost

during the reduction, possibly by some exchange process whilst the propyne was coordinated to the metal. Attempts to produce the *cis*-isomer using a Zn/Cu couple<sup>13</sup>, also monitored by GLC using the same conditions as above, showed that approximately 17 days were required to achieve 50% conversion of the propyne. As neither of these methods were effective an alternative approach was attempted using diborane in THF as the reductant<sup>14</sup>. Scheme 4 outlines the approach that we hoped to take: this involved cleavage of the propyne-borane adduct with deuterated acetic acid to give *trans*-[1-<sup>2</sup>H]propene. Using [1-<sup>2</sup>H]propyne instead of propyne and cleaving its borane adduct with acetic acid should give the *cis*-[1-<sup>2</sup>H]isomer. Attempts to produce the *cis*-isomer by this route gave a mixture containing the product and a large amount of unreacted propyne. As propyne is a good inhibitor of the enzyme this had to be removed. To overcome this problem the reaction was repeated and unreacted propyne was pumped off before the adduct was cleaved. No products were isolated from this attempt, possibly due to a reversible reaction of borane with propyne. It would have been possible to separate a propyne/propene mixture by preparative GLC but in order to avoid this, the method of Khanh<sup>9</sup> was used. This involved the formation of the Grignard reagent of *cis*-bromopropene, which was subsequently hydrolysed with <sup>2</sup>H<sub>2</sub>O to give the product, *cis*-[1-<sup>2</sup>H]propene. The *cis*-bromopropene was kindly donated by Dr. C. Pierpoint, whose method of production from the *trans*-but-2-enoic acid is reputed to give approximately 10% *trans*-bromopropene as by-product<sup>15</sup>. However, analysis of our product by <sup>2</sup>H NMR spectroscopy showed only a single isomer (a peak at  $\delta$  5.06).

Scheme 4.1 Proposed synthesis of *cis*- and *trans*-[1-<sup>2</sup>H]propene



4.6 EXPERIMENTALPreparation of Valeric Anhydride

The method used was that of Vogel<sup>16</sup>. The product was a colourless liquid that distilled at 88-90°C at 10 mmHg (lit. value 218°C at 754 mmHg)<sup>17</sup>.

<sup>1</sup>H NMR (CCl<sub>4</sub>, p.p.m.): 0.9 (t, 3 H), 1.4 (m, 2 H), 1.6 (m, 2 H), 2.4 (t, 2 H).

Main I.R. peaks (cm<sup>-1</sup>): 2960, 2860, 1820, 1750, 1460, 1030.

Preparation of N-propargylvaleramide

Propargylamine hydrochloride (0.49 g,  $5.4 \times 10^{-3}$  mol) was ground to a fine powder and suspended in pyridine (5 cm<sup>3</sup>). To this stirred suspension was added valeric anhydride (1.1 cm<sup>3</sup>,  $5.4 \times 10^{-3}$  mol) and stirring continued. After ca. 5 mins. the suspension cleared and a heavy precipitate collected at the bottom of the flask. Pyridine was removed on a rotary evaporator and the resultant yellowish residue was taken up in dichloromethane (20 cm<sup>3</sup>), washed with 2 M HCl (2 x 20 cm<sup>3</sup>), saturated sodium bicarbonate solution (2 x 20 cm<sup>3</sup>) and finally brine (10 cm<sup>3</sup>). The dichloromethane was dried (MgSO<sub>4</sub>), filtered and the solvent removed on a rotary evaporator leaving a yellowish-white residue. The residue was recrystallised from petrol-ether (60-80 fraction) yielding 0.3 g ( $2.1 \times 10^{-3}$  mol, 38.9% yield) of white crystals, m.p. 40-40.5°C, pure by t.l.c. analysis (Et<sub>2</sub>O, silica gel, iodine, R<sub>f</sub> 0.29).

<sup>1</sup>H NMR (CCl<sub>4</sub>, p.p.m.): 0.9 (t, 3 H), 1.35 (m, 2 H), 1.59 (m, 2 H), 2.05 (t, 1 H), 2.13 (t, 2 H), 3.95 (m, 2 H).

Main I.R. peaks ( $\text{cm}^{-1}$ ): 3280, 2960, 2920, 2860, 1640, 1550, 1460, 1360.

M.S. (electron impact,  $m/z$ ): 139 ( $M^+$  ion), 110, 97, 96 (base peak), 57, 55.

Micro-analysis: Calculated C, 69.06; H, 9.35; N, 10.07. Found C, 69.29; H, 9.38, N, 10.00.

#### Preparation of [1-<sup>2</sup>H]propyne

Liquid ammonia (ca. 375  $\text{cm}^3$ ) was distilled from sodium into a dry three necked, round bottomed flask fitted with an acetone/dry-ice condenser, a nitrogen inlet and a stirrer bar, all protected from moisture by a soda-lime drying tube. Whilst under a stream of  $\text{N}_2$  anhydrous ferric chloride (0.245 g,  $1.5 \times 10^{-3}$  mol) was added. This was followed by freshly cut sodium metal (6.24 g, 0.27 mol) which was added in small portions over a period of 30 mins. with vigorous stirring. Propyne (15.1 g, 0.38 mol) was then bubbled into the stirred mixture and the ammonia was allowed to evaporate overnight. Residual ammonia was removed by heating at reduced pressure (0.01 mmHg, oil bath  $80^\circ\text{C}$ ) and the resultant grey coloured powder was stored in a glove box. Sodium propyne (8.07 g, 0.13 mol) was placed in a dry round bottomed flask and  $^2\text{H}_2\text{O}$  (12  $\text{cm}^3$ , 0.6 mol) was added dropwise from a pressure equalising dropping funnel and the resultant gas was collected by displacement of water.

$^1\text{H}$  NMR ( $\text{C}_6\text{D}_6$ , p.p.m.): 1.4 (s, 3 H), 1.65 (m, residual, undeuterated signal).  
[The integral indicated ca. 83%  $^2\text{H}$  incorporation.]

### Preparation of *cis*-[1-<sup>2</sup>H]propene<sup>9</sup>

A dry, 2-necked round-bottomed flask was fitted with a coil condenser (which was protected by a silica gel drying tube), and a pressure equalising dropping funnel. The flask contained a stirrer bar and magnesium turnings (0.5 g,  $20.6 \times 10^{-3}$  mol) under THF (ca. 20 cm<sup>3</sup>). A crystal of iodine was added. *Cis*-bromopropene (1.96 g,  $16.2 \times 10^{-3}$  mol) was then added in small aliquots, with vigorous heating and stirring, which were discontinued when the formation of Grignard reagent reaction began. Finally, the reaction was allowed to proceed to completion (ca. 15 min.). Under a slow N<sub>2</sub> stream the Grignard complex was hydrolysed by <sup>2</sup>H<sub>2</sub>O, and after passage through a trap cooled by an ice-salt mixture, the gaseous product was collected in a trap cooled to liquid nitrogen temperature. The trap was removed and connected to an apparatus (see Fig. 4.6) which, after slow warming of the trap to room temperature, allowed the product to be collected as a gas by water displacement. This gave ca. 300 cm<sup>3</sup> gaseous product ( $13.4 \times 10^{-3}$  mol, 83% yield).

<sup>1</sup>H NMR (CCl<sub>4</sub> p.p.m.): 1.7 (d, 3 H), 4.89 (d, 1 H, *J*-ca. 11 Hz), 5.0 (residual, undeuterated signal), 5.75 (m, 1 H). [The integral indicates ca. 91% <sup>2</sup>H incorporation.]

<sup>2</sup>H NMR (C<sub>6</sub>H<sub>6</sub> p.p.m.): 5.1 (single peak).

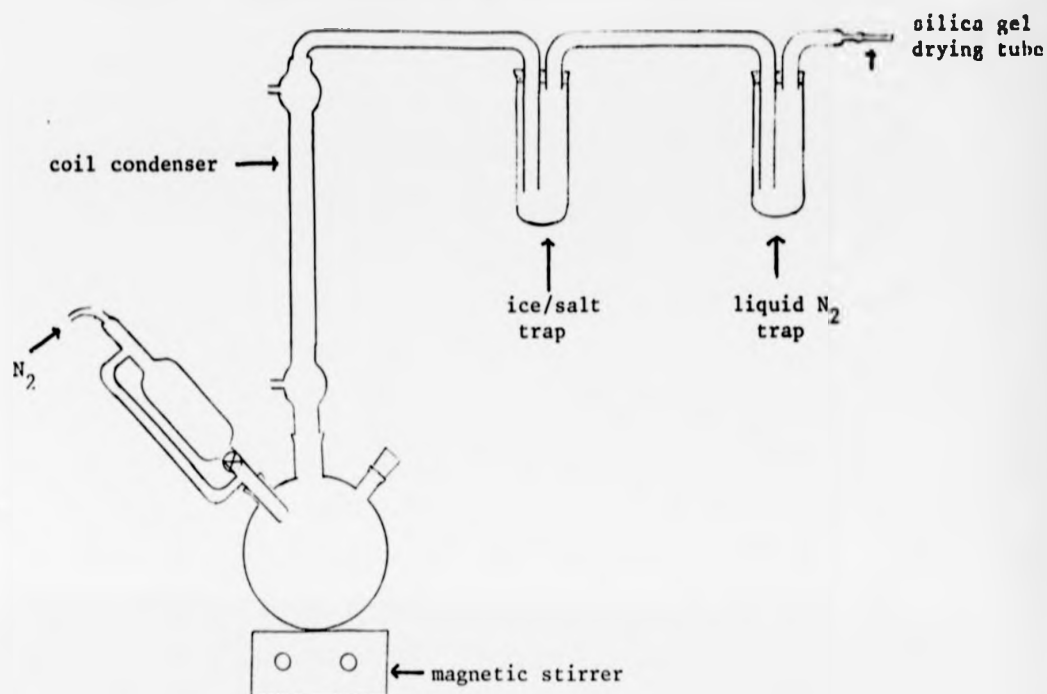
I.R. major peaks (cm<sup>-1</sup>): 3050, 2950, 2260, 1680, 1620, 1425

As this was a gas phase spectrum, considerable fine structure was seen.

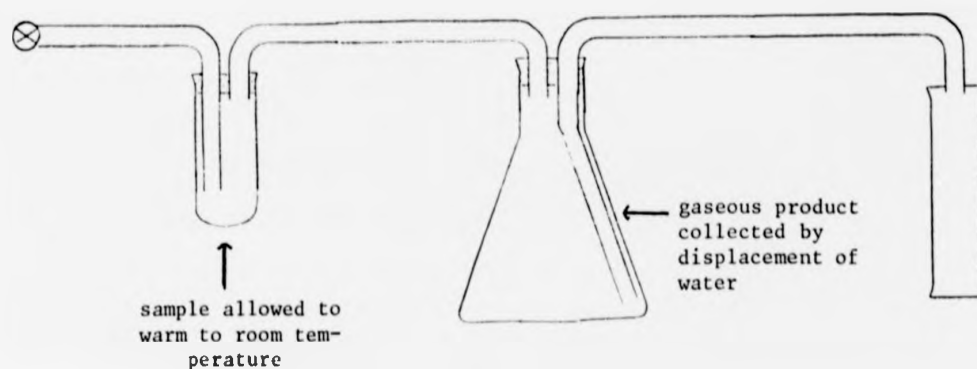
### Preparation of Cr(II) Reducing Agent<sup>18</sup>

Dilute copper(II) sulphate solution (3 drops) was added to zinc turnings (15 g), which were covered with 1 M HCl. When the

Fig. 4.6 Apparatus used for the deuteration of propene



Apparatus for the hydrolysis of the propyl-Grignard adduct



Apparatus for collection of gaseous product

zinc surface had turned black the HCl was replaced with saturated mercuric chloride solution, which was removed by decantation after the metal was fully coated with mercury, *ca.* 10 min. Mercury ( $50\text{ cm}^3$ ), followed by sufficient 1 M HCl to cover the metallic mass, was added and left for 6 hours, when all the zinc had dissolved.

Chromic potassium sulphate (100 g) was dissolved in 3 M HCl ( $250\text{ cm}^3$ ) and  $50\text{ cm}^3$  of the Zn/Hg amalgam was added. The contents of the flask were shaken under an atmosphere of  $\text{CO}_2$  until the solution turned from a dark blue to the translucent blue of  $\text{Cr}^{\text{II}}$  solutions.

#### Preparation of Zn/Cu Couple<sup>18</sup>

Copper sulphate (13 g) was dissolved in water ( $200\text{ cm}^3$ ) and the resultant solution stirred rapidly. Zinc dust (53.5 g) was added and stirring was continued for a further 15 min. when the black Zn/Cu couple was removed by filtration and washed with a dilute copper sulphate solution.

#### Preparation of 1-Methylcyclohexene<sup>19</sup>

Magnesium turnings (2.7 g, 0.11 mol) were placed in a dry, 2 necked round bottomed flask fitted with a reflux condenser, protected with a silica gel drying tube, a pressure equalising dropping funnel and a stirrer bar. The magnesium turnings were covered with diethyl ether ( $50\text{ cm}^3$ ) and methyl iodide (15.6 g, 0.11 mol) was added dropwise. The contents of the flask were stirred for 30 min. and cyclohexanone (8.66 g,  $90 \times 10^{-3}\text{ mol}$ ) in diethyl ether ( $10\text{ cm}^3$ ) was added dropwise over a 30 min. period.

Stirring was continued for 1 hour after which the flask was cooled and its contents poured onto a mixture of ice (60 g) and conc.  $\text{H}_2\text{SO}_4$  ( $2 \text{ cm}^3$ ). Diethyl ether was added until the precipitate dissolved, when the ether layer was removed and washed with 5%  $\text{H}_2\text{SO}_4$  ( $2 \times 20 \text{ cm}^3$ ), dried ( $\text{MgSO}_4$ ), filtered and the solvent removed on a rotary evaporator. The product was then distilled ( $58-59^\circ\text{C}$  at 16 mmHg) to give 7.2 g ( $63 \times 10^{-3} \text{ mol}$ , 70% yield) of a colourless liquid which was sufficiently pure by  $^1\text{H}$  NMR to be used directly for the next stage.

$^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.) 1.15 (s, 3 H), ca. 1.35 (complex m, 10 H), 3.7 (broad s, 1 H).

To this methylcyclohexanol (3 g,  $27 \times 10^{-3} \text{ mol}$ ), heated at  $110^\circ\text{C}$  in a round bottomed flask fitted with a reflux condenser and a magnetic stirrer, was added a few crystals of hydrated sodium acetate. Diketene (2.68 g,  $31.9 \times 10^{-3} \text{ mol}$ ) was then added dropwise over a 15 min. period during which the contents of the flask darkened in colour and refluxed gently. Stirring was continued for 3 hours at  $100^\circ\text{C}$  after which the product was Kugelrohr distilled ( $100^\circ\text{C}$  at 1 mmHg) and p-toluenesulphonic acid (16 mg) was added. The mixture was then heated at  $130^\circ\text{C}$  and the temperature gradually increased to  $180^\circ\text{C}$  at which temperature all of the product had distilled over. This product was redistilled ( $110-112^\circ\text{C}$ ) to give 1.32 g ( $13.8 \times 10^{-3} \text{ mol}$ , 50.9% yield) of a colourless liquid.  $^1\text{H}$  NMR ( $\text{CCl}_4$ , p.p.m.): 1.55 (m, 4H), 1.6 (s, 3 H), 1.9 (m, 4 H), 5.3 (m, 1 H).

#### Preparation of 2-cyclohexenol

2-Cyclohexenone (2.33 g,  $24.75 \times 10^{-3} \text{ mol}$ ) was dissolved

in dry ether (25 cm<sup>3</sup>) and stirred under a N<sub>2</sub> atmosphere for 20 min. and then cooled in ice.

Lithium aluminium hydride (0.35 g,  $9.2 \times 10^{-3}$  mol) was added to dry diethyl ether (25 cm<sup>3</sup>), which was heated at reflux temperature, with stirring under N<sub>2</sub> for 10 min. The mixture was cooled and added to the cyclohexenone solution dropwise over a 25 min. period to give a milky suspension which was stirred at room temperature for 18 hours. Acetone (3 cm<sup>3</sup>) was added and the mixture stirred for 3 min. after which a saturated solution of ammonium chloride (112 cm<sup>3</sup>) was added. The suspension was filtered and the filtrate was extracted with diethyl ether (4 x 25 cm<sup>3</sup>). The combined ethereal fractions were washed with brine (25 cm<sup>3</sup>), dried (MgSO<sub>4</sub>), filtered and the solvent removed on a rotary evaporator. The product was taken up in dichloromethane (5 cm<sup>3</sup>) and chromatographed on a short neutral alumina column. The solvent was removed on a rotary evaporator and the product was distilled (162-164°C) to give 1.09 g ( $11.1 \times 10^{-3}$  mol, 45.8% yield) of a colourless liquid.

<sup>1</sup>H NMR (CCl<sub>4</sub>, p.p.m.): 1.52 (m, 2 H), 1.75 (m, 2 H), 1.92 (m, 2 H), 4.05 (m, 1 H), 5.65 (m, 1 H).

## CHAPTER 4 - REFERENCES

1. J. Colby, D. I. Stirling and H. Dalton, *Biochem. J.*, 1977, 165, 395
2. D. I. Stirling and H. Dalton, *Arch. Microbiol.*, 1977, 114, 71
3. J. March, "Advanced Organic Chemistry", 2nd Ed., McGraw-Hill, 1977, and references cited therein.
4. G. A. Hamilton, R. J. Workman and L. Woo, *J. Am. Chem. Soc.*, 1964, 86, 3390
5. *Idem. Ibid.*, 1964, 86, 3391
6. K. B. Sharpless and T. C. Flood, *J. Am. Chem. Soc.*, 1971, 93, 2316
7. "Comprehensive Organic Chemistry", Vol. 1, D. Wern, 1979, Pergamon Press, and references cited therein
8. S. W. May, S. L. Gordon and M. S. Stellenkamp, *J. Am. Chem. Soc.*, 1977, 99, 2017
9. P. H. Khanh, T. Hirano and T. Tsurata, *J. Macromol. Sci. Chem.*, 1971, 5, 1287
10. A. K. Wong, M.Sc. Thesis, Warwick University
11. P. R. Ortiz de Montellano and K. L. Kunze, *J. Am. Chem. Soc.*, 1980, 102, 7375
12. H. Dalton, personal communication
13. P. P. Nicholas and R. T. Carroll, *J. Org. Chem.*, 1968, 33, 2345
14. "Organic Synthesis via Boranes", H. C. Brown, 1975, Wiley Interscience, and references cited therein
15. B. Mechini and N. Naulet, *J. Organomet. Chem.*, 1972, 39, 229
16. "Textbook of Practical Organic Chemistry", 4th Edn., Vogel, 1978, Longman, and references cited therein
17. "CRC Handbook of Tables for Organic Compound Identification", 3rd Edn., CRC Press
18. Ph.D. Thesis, D. C. Billington, 1979, Warwick University
19. C. Frisell and S. O. Lawesson, *Arkiv. fur Kemt.*, 1961, 17, 401
20. B. T. Golding, C. S. Sell, P. J. Sellars, *J. Chem. Soc. Perkin Trans. II*, 1980, 961



## CHAPTER 5

### CONCLUSIONS AND FUTURE WORK

Oxidation of substituted aromatic compounds by the M.C. monooxygenase, to give predominantly, if not exclusively, products of para-hydroxylation indicates the electrophilic nature of the 'active oxygen' species (see Chapter 3, Arenes). Similar results have been found in oxidations mediated by cytochrome  $P_{450}$  where both radical and direct insertion mechanisms have been proposed (see Chapter 1, Cytochrome  $P_{450}$ ). The failure to observe any ring-opened products arising from the oxidation of methylcyclopropane by the M.C. monooxygenase, would appear to preclude a radical mechanism in this case. Similarly, the oxidation of cyclopropane to give exclusively cyclopropanol would suggest that a mechanism involving a carbenium ion is also unlikely. All of these results are consistent with an oxenoid mechanism that proceeds *via* a direct insertion of the oxygen atom into a C-H bond (see Chapter 1, Models).

We have shown that oxidation of the substituent group on the aromatic ring occurs almost exclusively in the position  $\alpha$  to the aromatic ring. Chemically this pattern would be expected if a radical or charged intermediate were produced enabling stabilisation by the adjacent aromatic ring. The only exception to this regio-specificity that we found, in oxidations of aromatic side chains, was in the oxidation of  $\beta$ -methylstyrene, where oxidation was found to occur on the carbon distal from the aromatic ring. As this position is stabilised by conjugation with the aromatic ring then this result may not be as anomalous as it first appears. However, if such stabilisation of an intermediate is necessary then one would predict

that aliphatic compounds, particularly methane, would not be oxidised.

One possible explanation for these results could be the presence of more than one monooxygenase enzyme, cf. liver microsomal cytochrome P<sub>450</sub>. It is conceivable that in such a case the different monooxygenase enzymes may have different substrate specificities and possibly different mechanisms. If this were also the case with *Methylobacillus trichosporium* it could explain why Higgins and Stirling obtained such differing results for substrate specificities and electron donor characteristics (see Chapter 1, Bacterial Monooxygenases).

A considerable amount of work has been carried out by H. Dalton and his colleagues on the purification of the monooxygenase from M.C. and they have found no evidence to suggest the presence of more than one isoenzyme. In their purification procedures, however, they monitor the purity of the enzyme by means of aliphatic oxidation and it is possible that this may lead to the loss of any other isoenzyme present.

An alternative explanation for the regiospecificities seen in oxidations with the M.C. enzyme could be one based on steric fit to an active site. The suggestion made in Chapter 3 that the active site may be a narrow cleft or crevice in the surface of the protein could explain the exclusive para-hydroxylation of aromatic compounds. It is difficult, however, to see how such a model would predict the regiospecificities observed in the side chain oxidations. It was suggested that a long flexible or bulky side chain may prevent a good fit into the active site and that inclusion of a conjugated double bond into the side chain would decrease the number of conformers available and give a better 'fit'. Again, this explanation would predict that long chain alkanes such as hexane would be poor substrates, but they are found to be oxidised relatively efficiently.

In Chapter 3 it was suggested that the lack of side chain

oxidation of  $\alpha$ -methylstyrene could have been due to the steric inhibition mentioned above; in this case oxidation of the aromatic ring was observed. If this failure to oxidise the allylic region is due solely to steric reasons then one would predict that the unhindered end of the 2-methylbutenes would be oxidised, particularly as both *cis* and *trans*-2-butene are oxidised. The fact that there was no oxidation of the 2-methylbutenes argues against such a simplistic model.

As was mentioned above the regiospecificity observed in the oxidation of aromatic compounds suggests an electrophilic oxidising species. The failure of catalase and superoxide dismutase to prevent oxidations by the M.C. enzyme, assuming that hydroxyl radical is not involved for the reasons outlined in Chapter 1, suggests an iron-oxygen species as the active oxidant. This is consistent with the type of species believed to be involved in the oxenoid pathway, and the fact that an 'NIH shift' is seen in aromatic oxidations, lends support to this postulated pathway.

A major difference between the results found with the M.C. monooxygenase and other similar enzymes, e.g. cytochrome P<sub>450</sub>, is the fate of the arene oxide produced in aromatic oxidations. Most other monooxygenase enzymes give almost exclusively 1-naphthol from naphthalene oxidation. This is believed to arise from spontaneous ring opening of naphthalene-1,2-oxide. The presence of relatively large quantities of 2-naphthol in oxidations of naphthalene by the M.C. monooxygenase suggests that a similar spontaneous ring opening does not occur. As evidence suggests that naphthalene-2,3-oxide is not involved it is envisaged that some form of ring opening of the arene oxide by the enzyme occurs in M.C. mediated oxidations of naphthalene (see Chapter 3, Arenes).

The final evidence for this oxenoid mechanism comes from the oxidation of *cis*-[1-<sup>2</sup>H]propene to give a product propylene oxide in which geometrical configuration is retained. There is a need for the oxidation of *trans*-[1-<sup>2</sup>H]propene to be carried out to complete the above work. Also, the degree of deuterium retention for the oxidation of [4-<sup>2</sup>H]ethylbenzene, [1-<sup>2</sup>H]- and [2-<sup>2</sup>H]naphthalenes should be accurately determined. Further evidence for the intermediacy of naphthalene-1,2-oxide in the production of both 1- and 2-naphthols could be obtained by incubation of an authentic sample of naphthalene-1,2-oxide with the enzyme mixture.

Finally, oxidation of a chiral methyl group, (Chapter 1, Cytochrome P<sub>450</sub>) should help substantiate the belief that a direct insertion pathway is the mechanism used by the M.C. monooxygenase.

## CHAPTER 6

### MATERIALS, METHODS AND INSTRUMENTATION

#### 6.1 MATERIALS AND METHODS

Unless otherwise stated, all solvents and chemicals used were of analytic grade or were purified by distillation. Anhydrous solvents were prepared as follows.

##### Diethyl Ether

AR diethyl ether was heated to reflux over, and fractionally distilled from  $\text{LiAlH}_4$ , under a stream of nitrogen, b.p.  $35^\circ\text{C}$ .

##### Pyridine

AR pyridine was heated to reflux with KOH pellets for 1 hour, fractional distillation gave anhydrous pyridine (b.p.  $115-116^\circ\text{C}$ ), stored over KOH.

##### Dichloromethane

Dichloromethane was distilled from calcium hydride b.p.  $40^\circ\text{C}$ .

##### Dimethylsulphoxide

Heated to reflux over  $\text{CaH}_2$  for 2 hours followed by fractional distillation b.p.  $85-87^\circ\text{C}$ .

##### Petrol Ether

Shaken with conc.  $\text{H}_2\text{SO}_4$  followed by water, sodium bicar-

bonate and again water, dried with calcium chloride and then fractionally distilled.

#### Chloroform

Ethanol-free chloroform was prepared by passing AR chloroform down a short alumina column.

#### NADH

NADH was purified by dissolving in water to give the required concentration. This was then extracted with diethyl ether to remove stabilising ethanol, and the resultant solution placed on a rotary evaporator to remove residual diethyl ether.

#### Water

In an attempt to remove the traces of acetone mentioned previously (Chapter 2, cyclopropanes) and any other trace impurities, doubly distilled water was heated at reflux temperature with alkaline  $\text{KMnO}_4$  for 24 hours. The water was then fractionally distilled twice, but g.l.c. analysis still showed the presence of a trace impurity that co-chromatographed with acetone.

#### Preparative t.l.c. plates

As mentioned in the text (e.g. page 96), the  $^1\text{H}$  n.m.r. spectroscopic analyses of the products of enzymic oxidations were often complicated by resonances from trace impurities. To minimise these problems, either AR grade solvents were used or the solvents were purified as described earlier in this Chapter (page 140). The

preparative t.l.c. plates used for purification of the products were first eluted with 10% MeOH in  $\text{CH}_2\text{Cl}_2$ , dried and reactivated before use.

#### Enzymic Oxidations

Enzymic oxidations were carried out on two different scales, the smaller scale oxidation being used as an analytic run to see if oxidation had occurred. A number of controls were used including the inclusion of acetylene (a known inhibitor of the M.C. monooxygenase) and cyanide which inhibits the alcohol dehydrogenase but not the monooxygenase.

#### Experimental

For oxidations of solid or liquid compounds five  $5\text{ cm}^3$  conical flasks were used and the contents of each is listed below.

- Flask 1    Phosphate buffer, 20 mmolar, pH 7, ( $0.4\text{ cm}^3$ ),  
            substrate ( $5 \times 10^{-3}\text{ g}$ )
- Flask 2    Phosphate buffer, 20 mmolar, pH 7, ( $0.4\text{ cm}^3$ ),  
            substrate ( $5 \times 10^{-3}\text{ g}$ )
- Flask 3    Phosphate buffer, 20 mmolar, pH 7, ( $0.4\text{ cm}^3$ )
- Flask 4    Phosphate buffer, 20 mmolar, pH 7, ( $0.4\text{ cm}^3$ ),  
            substrate ( $5 \times 10^{-3}\text{ g}$ ), KCN solution, 0.5 mmolar  
            ( $5 \times 10^{-6}\text{ cm}^3$ )

Flask 5    Phosphate buffer, 20 mmolar, pH 7, ( $0.4 \text{ cm}^3$ ),  
              substrate ( $5 \times 10^{-3} \text{ g}$ )

The flasks were then suba sealed and to flask 5 one  $\text{cm}^3$  of acetylene was added from a syringe displacing an equivalent amount of air. The flasks were then warmed to  $45^\circ\text{C}$  on a shaking water bath and M.C. extract ( $100 \times 10^{-3} \text{ cm}^3$ ) was added by syringe, along with NADH solution 0.1 molar ( $50 \times 10^{-3} \text{ cm}^3$ ). No NADH solution was added to flask 2. The flasks were then returned to the shaking water bath for 60 minutes after which they were extracted with  $\text{CH}_2\text{Cl}_2$  ( $1 \text{ cm}^3$ ) which was removed, dried ( $\text{MgSO}_4$ ), filtered and concentrated to ca.  $100 \times 10^{-3} \text{ cm}^3$  with a stream of nitrogen. The contents of the flasks were then chromatographically analysed.

For gaseous or volatile substrates the same procedure was used with the exception that the substrate ( $1 \text{ cm}^3$  of vapour) was added by syringe after the flasks were suba sealed. Also the  $\text{CH}_2\text{Cl}_2$  used to extract the products was added by syringe before the suba seal was removed.

Larger scale oxidations designed to give sufficient product for spectroscopic analysis (ca. 2% conversion) were carried out as follows.

A  $250 \text{ cm}^3$  conical flask was charged with phosphate buffer, 20 mmolar, pH 7 ( $8 \text{ cm}^3$ ) and 0.1 g of substrate. The flask was then suba sealed and warmed to  $45^\circ\text{C}$  on a shaking water bath. NADH solution, 0.1 molar ( $1 \text{ cm}^3$ ) was then added along with crude extract ( $2 \text{ cm}^3$ ) and the flask was returned to the water bath for 1 hour. The contents of the flask were then extracted with  $\text{CH}_2\text{Cl}_2$  ( $3 \times 20 \text{ cm}^3$ ) with centrifugation, where necessary, to assist separation of the layers. The  $\text{CH}_2\text{Cl}_2$  aliquots were then dried ( $\text{MgSO}_4$ ), filtered and the solvent removed by rotary evaporator. The product was then purified by chromatography. If the substrates were volatile or gaseous



the reaction was carried out in a 50 cm<sup>3</sup> conical flask and 6 cm<sup>3</sup> of the vapour was added by syringe. Dichloromethane was added to extract the products before the suba seal was removed.

Exceptions to the above work up procedure were as follows: in the case of the oxidation of methylcyclopropane continuous extraction by diethyl ether (100 cm<sup>3</sup>) was required for a period of 48 hours; after the oxidation of [1-<sup>2</sup>H]propene, benzene (1 cm<sup>3</sup>) was added, the product was extracted and the benzene layer separated, dried (MgSO<sub>4</sub>), filtered and analysed by <sup>1</sup>H and <sup>2</sup>H NMR directly.

It is possible that products from the enzymic oxidations undergo further oxidation or some other type of reaction. For cytochrome P<sub>450</sub> it has been argued\* that a second oxidation is unlikely. This is because once the product has been released from the active site it would be present in a relatively large pool of substrate and hence statistically unlikely to encounter the enzyme a second time. Also, it has been argued that incorporation of an oxygen atom into the substrate will increase its polarity and consequently decrease its affinity for the hydrophobic active site. The first argument is relevant to the M.C. enzyme, particularly as the conversions of substrate to product are generally low (ca. 1%). No products were ever seen on t.l.c. analysis that could be ascribed to further oxidation.

Where possible the enzymic products were also analysed by mass spectroscopy and the resultant spectra compared with those obtained from authentic samples of the compounds. This, however, was of limited use due to the impurities in the products giving rise to anomalous peaks in the spectra.

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\*R. E. White and M. J. Coon, *Ann. Rev. Biochem.*, 1980, 49, 315

## 6.2 INSTRUMENTAL

### NMR Spectra

$^1\text{H}$  NMR spectra were recorded by the author using a Perkin Elmer (model R34) 220 MHz  $^1\text{H}$  NMR spectrometer.

Peaks are designated by their chemical shift(s) in p.p.m., relative to TMS, followed by their relative integral value (eq. 1 H, 2 H), and their multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet).

$^2\text{H}$  NMR spectra were recorded by Dr. E. Curzon using a Bruker (model WH400), 400 MHz multinuclear NMR spectrometer.

### IR Spectra

Infra-red spectra were recorded on a Perkin Elmer (model 680B) grating infra-red spectrophotometer. Spectra were run using NaCl plates and calibrated using the  $1603.4\text{ cm}^{-1}$  peak of polystyrene. Samples of liquids were run as thin films and solids were run as a mull (nujol).

### Ultra-violet Spectra

U.V. spectra were recorded using a Cecil (model CE505) double beam spectrophotometer with spectroscopic grade methanol as solvent, and a holmium filter as reference.

### Gas Liquid Chromatography

GLC analyses were carried out using either a Pye Unicam (model 204) or a Perkin Elmer (model F11) flame ionisation chromatograph with  $\text{N}_2$  as carrier gas.

Preparative GLC was carried out on either a F & M (model 720) catharometer, or a Carlo Erba (model 2450 TP) flame ionisation, gas chromatograph with  $N_2$  as the carrier gas.

#### Mass Spectra

Mass spectra were recorded on a Carlo Erba-Kratos instrument (MS80). Peaks are quoted as  $m/z$ .